Treatment of Acute Leukemias

New Directions for Clinical Research

EDITED BY

Ching-Hon Pui, MD

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PREFACE

The impetus for *Treatment of Acute Leukemias: New Directions for Clinical Research* came from many conversations with colleagues and from my years of patient care experience at St. Jude Children's Research Hospital in Memphis, TN. The message was clear—too often we rely on discoveries in the laboratory to drive the next wave of treatment advances when, in fact, substantial progress can be made by identifying and discussing pivotal issues that might be resolved through better application of current methods of leukemia management. Although evolving insights from molecular biology studies are certain to translate into improved therapies directed at specific and unique targets, we still need to care for patients who cannot wait for these developments. Thus, I invited pairs of international experts to address 21 topics that continue to challenge clinical researchers who treat leukemia. These authors were asked to provide expert commentary in lieu of exhaustive descriptions of published studies. My hope is that these dual points of view have achieved a broad and balanced perspective on each topic.

A book of this type almost always contains some redundancies because of the need for completeness within single chapters, and the leukemia-related terminology tends to vary among subdisciplines and even among research groups. Nonetheless, I feel confident that such flaws have not detracted from the overall aim of the book, which was to compile the major debates that surround leukemia therapy at the beginning of the new millennium.

Part I focuses on the advantages and disadvantages of extant leukemia classification systems and the need for a single international system that incorporates the best features of each. Both chapters recognize the overriding importance of genetic risk factors, especially those that directly influence response to therapy, and devote considerable attention to how these emerging factors can be used to guide treatment selection and predict clinical outcome. Owing to the continuing rapid advances in technology and the development of more robust methods of microarray analysis, conventional immunophenotyping and genotyping may soon be replaced by gene expression profiling.

Part II, Chemotherapeutic Strategies, is the heart of the book and covers accepted and experimental treatments of the main forms of acute leukemia in children and adults. We learn in Chapters 3 and 4 that although acute lymphoblastic leukemia (ALL) in infants constitutes only 3% of childhood ALL cases, infant ALL warrants special consideration because of its unique constellation of features and resistance to standard therapy. Both authors agree that there is a need for "hybrid" treatment regimens for this leukemia variant and for greater international cooperation in evaluating such regimens in controlled clinical trials.

Three of every four cases of childhood acute leukemia are ALL; hence, this subtype is the focus of intense investigation by many independent research centers and cooperative study groups. Chapter 5 identifies six specific areas of controversy in the treatment of childhood ALL, including the relevance of residual disease measurements and the indications for stem cell transplantation during first complete remission. Chapter 6 adds alternative points of view to each of these debates and includes a final section on the true definition of treatment success, that is, whether a successful outcome should be defined solely on the basis of the long-held gold standard, event-free survival, or should include measures of quality-adjusted overall survival.

Adolescents and young adults are often treated arbitrarily on pediatric or adult protocols of chemotherapy, a fact that leads to diverse outcomes in these specific age groups. Chapters 7 and 8 argue convincingly that ALL cases in adolescents and young adults have a similar biology and tolerance to therapy, mandating more intensive chemotherapy than would generally be administered to older adult patients, as well as independent evaluation in multicenter clinical trials. In contrast to the high cure rates typically seen in childhood ALL, fewer than half of the adults with this disease achieve prolonged leukemia-free survival; this finding is mainly attributed to an increased frequency of the Philadelphia chromosome, a multidrug-resistance phenotype, and poor tolerance to therapy. As pointed out in Chapters 9 and 10, most of the controversial issues in adult ALL remain unresolved because of the lack of prospective, randomized multicenter trials. Nevertheless, the authors identify several promising strategies, such as wider use of high-dose cytarabine and stem cell transplantation, together with close monitoring of residual leukemia, which may lead to a better outcome in this historically poor prognostic group.

A decrease in the rate of central nervous system (CNS) relapse to 2% or lower in many recent studies has raised new questions about the CNS-directed treatment of childhood ALL, as adroitly outlined in Chapters 11 and 12. Most important, perhaps, is whether patients can be spared the hazards associated with cranial irradiation. The consensus opinion of these authors is that radiation-free treatments can be substituted in the vast majority of all newly diagnosed cases.

Chapters 13 and 14 focus exclusively on the challenges posed by the clinical management of relapsed ALL. The most urgent need, by far, is to identify methods that distinguish the subgroups that are likely to benefit from stem cell

transplantation from those who might be cured by intensive chemotherapy alone. The authors carefully evaluate numerous guidelines thought to be useful in this regard and suggest future directions, such as routine monitoring for residual leukemia, to discriminate among patients with a good, intermediate, or poor prognosis.

Mature B-cell ALL warrants separate coverage because of its distinctive features at diagnosis and unique treatment requirements. Despite the excellent cure rates achieved with high doses of cyclophosphamide, cytarabine, and methotrexate, for example, outstanding questions remain regarding the need for additional cytotoxic drugs, the optimal approach to CNS-directed therapy, and the role of supportive-care treatment such as uricolytic agents. Chapters 15 and 16 provide a critical analysis of these and other issues and remind us that the current therapy for B-cell ALL is both difficult to administer and highly toxic, justifying the efforts to devise new therapeutic strategies.

Although acute myeloid leukemia (AML) accounts for only 20% of cases of acute leukemia among children, it produces a disproportionate share of the leukemia-related mortality. Thus, the primary issue in the treatment of this disease concerns approaches that might improve historically inferior results. Chapters 17 and 18 evaluate strategies that hold the promise of optimizing available therapies, such as extending allogeneic stem cell transplantation to patients whose disease is not likely to respond to standard regimens of chemotherapy. These chapters also describe new directions that would avoid the excessive toxicity associated with many current protocols, including substitution of molecularly targeted agents. The even higher rates of relapse and death in cases of adult AML dictate innovative revisions of contemporary treatments. Chapters 19 and 20 call attention to the promising results of autologous and allogeneic stem cell transplantation in selected groups of patients, of antibody-based therapy, and of nonmyeloablative allogeneic transplantation in older patients with AML. Finally, Chapters 21 and 22 consider the unusual case of acute promyelocytic leukemia (APL). This AML subtype is exquisitely sensitive to all-*trans* retinoic acid, which induces benign differentiation of APL, and to arsenic compounds, which induce both apoptosis and differentiation. Retinoic acid-arsenic treatment of APL serves as a paradigm for the development of molecularly targeted therapy in acute leukemia and warrants the close scrutiny paid by these authors to mechanisms of drug action and optimal combinations of these agents within the context of standard APL treatment.

Only one in five children with acute leukemia who lives in underprivileged countries has access to adequate treatment, resulting in a long-term survival probability of less than 30% in these children. This sobering fact reminds us of the difficulty of translating therapeutic advances into protocols that benefit children worldwide. Chapters 23 and 24 describe how small but steady and consistent steps can be taken to remedy this situation and bring about dramatic change. The authors cite the successes gained by greater cooperation ("twinning") between pediatric centers in developing countries and those in developed countries and by stronger relationships between the medical staff members of hospitals in developing countries and their patients ("therapeutic alliances"). One remaining challenge is to define minimal treatments that will secure reasonable leukemia-free survival rates in nations with limited resources.

Part III examines the premise that many antileukemic drugs have unexploited potentials that could be harnessed to improve treatment outcome. Chapters 25 and 26 address issues that continue to impede optimal use of methotrexate. What are the most effective doses of "high-dose" methotrexate against specific cell lineages and genetic subtypes of ALL? What are the situations in which low doses of this drug are more effective than high doses? What are the clinically relevant mechanisms underlying resistance to methotrexate, and how can they be neutralized?

Although a mainstay of ALL therapy for over 20 years, L-asparaginase administration still has limitations, including the development of allergy, rapid clearance, induction of cellular resistance, and dose-limiting toxicity. Suggestions are made in Chapters 27 and 28 as to how these obstacles might be overcome. Particular emphasis is placed on the advantages of dose adaptations in individual patients, based on careful monitoring of pharmacologic end points. The drug 6-mercaptopurine and its analog 6-thioguanine have been used productively in so-called continuation therapy for nearly a half century, yet many questions remain concerning the optimal manner in which to incorporate these agents into multiagent protocols. As pointed out in Chapters 29 and 30, the results of pharmacogenetic studies can guide the optimal use of this class of agents.

The roles of etoposide and teniposide in acute leukemia therapy are highly controversial. Chapters 31 and 32 cast some doubt on the clinical utility of these compounds, citing their tendency to induce secondary AML and the lack of randomized trials to demonstrate that either epipodophyllotoxin can significantly improve outcome. The authors nonetheless identify the patients who appear to benefit most from these agents, as well as the drug dosages and schedules linked to acceptable levels of toxicity.

With the increasing range of donors and stem cell sources available to transplant specialists, one can look forward to wider use of hematopoietic stem cell transplantation in the treatment of acute leukemias. Thus, it is important to define the subgroups of patients for whom transplantation (but not chemotherapy alone) will provide a high likelihood of cure. Chapters 33 and 34 in Part IV offer expert opinions on this topic and on methods that can increase the efficacy and reduce the complications of this procedure.

Part V, Biologic Treatments, describes both the use of cytokines to rescue depleted bone marrow reserves and the administration of monoclonal antibodies, immunotoxins, donor lymphocytes, and activated T cells as antileukemic

therapy. In principle, treatment with the myeloid colony-stimulating factors G-CSF and GM-CSF could shorten the duration of neutropenia after intensive chemotherapy, leading to better protocol compliance and, possibly, to improvements in the long-term survival rates. However, as noted by the authors of Chapters 35 and 36, the results of clinical trials have not always supported this expectation, indicating limited applications of these growth factors in supportive care. There is much enthusiasm about the prospect of improving cure rates in acute leukemia through the use of immunotherapy. Chapters 37 and 38 critique recent studies of infusions of donor lymphocytes to enhance the graft-versus-leukemia effect of allogeneic transplantation, preliminary trials of antibody-based treatments, and experiments with activated syngeneic T cells in murine models.

Part VI takes a closer look at the assumption that a more complete understanding of drug resistance will lead to more effective treatments. All too often, it seems that cancer cells possess the ability to circumvent even the cleverest schemes of bypassing drug resistance. As discussed in Chapters 39 and 40, this conundrum results from the multifactorial nature of drug resistance and dictates a new focus on strategies that employ multiple agents to target specific pathways of growth, survival, and resistance. The direct corollary of drug resistance is minimal residual disease, whose clinical significance has been a topic of great interest and debate for at least 20 years. Thus, Part VII weighs the available evidence on the detection and monitoring of minimal residual disease and offers advice on the strategies that are best suited for use in the clinic.

The publication of this book would not have been possible without the generous support of St. Jude Children's Research Hospital, the American Lebanese Syrian Associated Charities (ALSAC), the American Cancer Society, and the National Cancer Institute (Cancer Center Support grant CA-21765). Special thanks are owed the authors, whose clinical perspectives will undoubtedly stimulate new interest in many long-standing issues. I dedicate this book to my parents and to all of the patients that I have been privileged to care for over the years.

Ching-Hon Pui, *MD*

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I

Diagnosis, Classification, and Prognosis

1 Classification of Acute Leukemias

Perspective 1

WOLF-DIETER LUDWIG, TORSTEN HAFERLACH, AND CLAUDIA SCHOCH

CONTENTS

1. INTRODUCTION

Research over the last two decades has demonstrated that acute leukemias, originally subdivided by morphologic and cytochemical criteria into myeloid and lymphoblastic varieties, represent highly heterogeneous groups of malignancies that for the most appropriate tailoring of therapy, require an extensive routine diagnostic workup, including immunophenotyping as well as cytogenetic and molecular genetic study. This more detailed characterization of leukemic blasts has provided information complementary to the classical morphology- and cytochemistry-based diagnosis and classification of acute leukemias in terms of our understanding of biologically and clinically relevant subsets; more recently, it has been successfully applied to the detection of minimal residual disease (MRD). Through the use of conventional and molecular cytogenetic analyses, acute leukemias have been recognized as a genetic disease, resulting from a series of acquired or inherited mutations in the structure of certain genes *(1)*. These mutations are passed from the original transformed progenitor cell to its clonal descendants. Most of the genetic aberrations fall into generic classes of functional dysregulation that subvert normal hematopoietic developmental programs by circumvention of cell cycle controls, inhibition of differentiation, and resistance to therapeutic apoptosis in leukemic blasts *(2)*.

The morphologic, cytochemical, and immunophenotypic criteria defined by the French–American–British (FAB) classification *(3–6)* still represent the gold standard for subclassification of acute myeloid leukemia (AML). On the basis of lineage commitment and the degree of blast cell differentiation, these criteria have led to the recognition of eight major AML subgroups (AML M0–M7). Given the distinct clinical behavior and response to treatment of the FAB categories, however, the identification of specific entities, defined according to a combination of morphologic, immunophenotypic and genetic features and clinical syndromes, has become a desirable goal. This objective, originally addressed by the so-called morphologic, immunologic, and cytogenetic (MIC) working classification of AML *(7)*, has been partly reached by the routine application of cytogenetics and molecular genetics to the initial characterization of AML. It has provided valuable insights into

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the pathogenesis of AML as well as treatment strategies targeting the underlying specific molecular abnormalities. Furthermore, a vast array of AML phenotypes and genotypes, which have been identified during the last decade, correlate to various degrees with each other, with the patient's presenting characteristics, and with the clinical behavior of the disease.

Unlike the FAB classification of AML, that of acute lymphoblastic leukemia (ALL) *(3)* has not been shown to have significant immunophenotypic, genetic, and clinical correlates, except for the L3 subtype, and therefore it has been largely replaced by immunophenotypic and genetic schemes. The accurate assignment of leukemic lymphoblasts to specific lineages has been made possible by the use of lineage- and/or maturation-specific monoclonal antibodies (MAbs); hence, the primary diagnosis and subclassification of ALL nowadays rest on immunophenotyping *(8,9)*. The last few years have seen a variety of novel genetic markers that provide crucial information for understanding the biology of both AML and ALL, that can be used as diagnostic and prognostic tools, and that give important clues to rational therapeutic interventions *(1,10)*.

Obviously, a gene-based classification system would be preferable to one relying mainly on indirect measures of blast cell diversity, such as morphology and immunophenotype. There is little doubt that new approaches to acute leukemia classification, such as gene expression profiling with DNA microarrays, will contribute importantly to the identification of acute leukemia subtypes with distinct clinical phenotypes and variable clinical courses *(11–13)*. Future studies should demonstrate whether the molecular classification of acute leukemias on the basis of gene expression profiling will in fact lead to a clinically relevant stratification of AML and ALL into molecularly defined categories and to the development of new therapeutics aimed at the correction of pathologic transcriptional programs.

In the following paragraphs, we start with some general considerations on the techniques currently applied to the classification of acute leukemias. Subsequently, the impact of morphologic and cytochemical analyses, immunophenotyping, and cyto-/molecular genetics for dissecting the cell-biologic heterogeneity of acute leukemias and for defining clinically relevant subsets is covered in greater detail. Finally, controversial issues in the classification of acute leukemias are discussed.

2. MORPHOLOGIC AND CYTOCHEMICAL ANALYSIS

For nearly 100 years, even in the present era of molecular biology, the classification of blood cells and the diagnosis of leukemia have been based on cytomorphologic features after staining. AML therapy depends mainly on the interpretation of the morphologic appearance of blasts in the microscope. Therefore it is necessary to describe the sources, staining techniques, and possible pitfalls. Cytomorphology should also lead to a rational use of the techniques described below, such as immunophenotyping, cytogenetics, fluorescence *in situ* hybridization (FISH), and the polymerase chain reaction (PCR). The morphologic diagnosis of acute leukemias should always be based on the examination of blood *and* bone marrow smears. If the aspiration of bone marrow fails (*punctio sicca*), the examination has to focus on the peripheral blood smears alone, taking into account the histologic results of a trephine biopsy. However, not all staining techniques are useful any more. Absolutely necessary are the May–Grünwald–Giemsa stain, the myeloperoxidase (MPO) reaction, and the nonspecific esterase (NSE) reaction. All other methods seem to be superfluous, especially when other techniques (such as immunophenotyping, cytogenetics, FISH, and molecular genetics) are applied. However, judicious use of the information derived from cytomorphology and cytochemistry allows better, faster, and more efficient use of the techniques described below.

2.1. Staining Techniques for the Diagnosis of Acute Leukemias

Although a detailed discussion of staining and cytochemistry protocols *(14,15)* is beyond the scope of this chapter, some information should be given on how to interpret staining results and avoid mistakes that lead to misdiagnosis. Standard hematologic staining techniques include May–Grünwald–Giemsa (MGG; or Pappenheim, Romanowsky, Wright, Wright-Giemsa). The information obtained by these simple methods leads directly to the diagnosis of AML (e.g., by the detection of Auer rods) or allows the well-directed decision to carry out further investigations. Baselines for the interpretation of a sufficient staining result should be established to permit comparisons with other laboratories. Blood smears and, even more importantly, bone marrow slides with occasional high cellularity and marrow particles should dry for at least 1–3 h for best staining results. Reproducible staining results with the MGG or Pappenheim stain are characterized by three features: (1) they should show erythrocytes in salmon color; (2) the granules in the polymorphonuclear neutrophils (PMNs) should be visible (not fulfilled with the Wright stain for bone marrow); and (3) the platelets should have red–blue colors. If this is not the case, the result of the PMN testing may be misinterpreted as hypogranular, which may lead to the erroneous diagnosis of dysgranulopoiesis.

Selective cytochemical stains improve the accuracy and reproducibility of lineage assessment and traditional AML subclassification. MPO, Sudan black B (SBB), and NSE with α-naphthyl acetate (ANA) or α-naphthyl butyrate (ANB) used as a substrate, are useful in this regard. In most cytochemical determinations of MPO, diaminobenzidine (in some investigations benzidine) is used. Blood films and bone marrow should not be older than 2–5 d for a reliable MPO reaction. Heat or sunlight destroys the cells even more quickly. Promyelocytes, bands, and PMNs show a yellowish to brownish granular stain, which appears strongest in eosinophils; monocytes may show a weak reaction. Lymphoid cells are always negative.

For the interpretation of blasts in AML and to determine peroxidase deficiency, it is always necessary to find some PMNs that are MPO-positive. Otherwise, the reaction may have failed. If this possibility can be excluded, and an MPO defect is detected in more than 50% of the PMNs, one is led to the diagnosis of myelodysplasia with MPO deficiency. In these cases, eosinophils are still positive.

The MPO reaction is still the morphologic gold standard for discriminating between myeloid and lymphoid blasts. Furthermore, Auer rods can be detected up to three times more often

by investigating the MPO reaction than by using MGG stain exclusively *(16)*. For SBB stain, interpretation of the positive reaction is very similar to that of the MPO reaction *(17)*. However, very weak reactivity will be detected better if the peroxidase reaction is used. SBB is reactive in unfixed, air-dried smears much longer than MPO. The detection of esterase is crucial for the definition of monoblasts or monocytes, especially when the cytomorphology in the MGG stain is not typical. For routine purposes, it is helpful to describe the reaction by identifying the diffuse staining of the cytoplasm on a scale from 0 to 4+ (no reaction to very strong reaction). Only when cells have been identified as 3+ or 4+ is it justifiable to describe them as monoblasts or monocytes. Mature monocytes always react less intensively than immature monoblasts. Cells from the monocytic compartment in the bone marrow show a stronger reaction than do monocytes on a peripheral blood smear. The reaction can be inhibited with sodium fluoride. As an internal control, megakaryocytes and macrophages react positively with ANA. Reactivity for ANA on unfixed, unstained smears is stable for weeks.

The NSE reaction is always necessary for AML classification in the FAB system as well as for the diagnosis of chronic myelomonocytic leukemia. Stains such as naphthol ASD-chloroacetate-esterase (specific esterase; CAE), periodic acid-Schiff (PAS), acid phosphatase, dipeptidylaminopeptidase IV (DAP IV), and iron have no further role for the diagnosis of acute leukemia on bone marrow or blood smears.

2.2. Morphologic Classifications of Acute Leukemias

The morphologic classification of acute leukemias requires peripheral blood *and* bone marrow smears (or touch preparations) for all patients. A number of peripheral cells and bone marrow cells (100–200) should be examined. Especially in ALL, the percentage of infiltration sometimes varies widely, and blasts can be found in clusters. The FAB classifications of acute leukemias *(3–6,18,19)* follow an algorithm and are based on several thresholds. In the era of biologic description of disease entities, some of these rules seem to be arbitrary, a limitation considered in the development of new proposals, such as the World Health Organization (WHO) classification discussed below *(20)*. However, the FAB system still forms the basis for the cytomorphologic classification of AML and MDS at diagnosis, but not of ALL. The definition of acute leukemia and the distinction between AML and ALL according to the FAB system are based on two criteria:

1. The percentage of blasts in the bone marrow is >30% of all nucleated cells
2. Three percent or more of blasts show a positive reaction for MPO or SBB in the bone marrow.

The definition of *complete remission* in the acute leukemias has been published by the Cancer and Leukemia Group B (CALGB) and includes the following criteria *(21)*.

1. Bone marrow blasts < 5%.
2. Neutrophils > 1500/ μL.
3. Platelet count > 150,000/μL.

Some other definitions include different thresholds for neutrophils (i.e., whole white blood cell count) and platelets (>100,000/μL). In most study groups, however, the CALGB criteria are accepted and should be standard until other criteria are available.

Measurement of the percentage of blasts in the bone marrow is sometimes difficult after chemotherapy. Immature granulopoietic cells in early regeneration have to be distinguished from remaining myeloid blasts. In critical cases, the cytomorphologic appearance at diagnosis and at remission induction should be analyzed in parallel. In some cases, a control biopsy should be performed after another 7 d of hematopoietic regeneration. For deeper insights into the remission status and MRD, parallel investigations should include cytomorphology, immunophenotyping, cytogenetics (including FISH), and molecular techniques.

3. IMMUNOPHENOTYPING

In the past two decades, the impact of immunophenotyping by flow cytometry in the diagnosis and management of acute leukemia has expanded rapidly. This advance can be attributed mainly to significant progress in laser and computer technologies, the production of several hundred MAbs to a variety of antigens expressed by hematopoietic cells, and the availability of distinct fluorochromes conjugated to MAbs, allowing the simultaneous measurement of at least three or four cellular antigens in combination with two intrinsic parameters (cell size and cytoplasmic complexity) as determined on the basis of the leukemic cells' light-scatter properties [i.e., forward- and side-scatter characteristics (FSC and SSC)] *(9,22–26)*. Given these technical achievements, immunophenotyping by multiparameter flow cytometry has emerged as an optimal method for the immunodiagnosis of hematopoietic maligancies and has largely replaced microscopic analysis with immunocytochemical techniques, because flow cytometry provides an objective, sensitive, and rapid multivariate analysis of high numbers of cells. Accordingly, there is now general consensus that multiparameter flow cytometry is a powerful diagnostic tool for the immunophenotypic characterization of acute leukemias and chronic lymphoproliferative disorders that can be applied to define immunophenotypic subsets, to detect MRD, and (more recently) to develop and monitor antibody-based treatment strategies *(9,22,23,26,27)*.

Most previous studies investigating the diagnostic impact of immunophenotyping and the association between antigen expression and treatment outcome in acute leukemias have used 20% of cells stained with MAbs for surface markers and 10% for more specific markers usually expressed in the cytoplasm (e.g., MPO, CD79α, cytoplasmic CD3) as the general cut points for marker positivity *(28)*. Obviously, these values were chosen arbitrarily and have been criticized *(29)*, since they are not based on physiologic knowledge but rather serve as a convenient means of data collection. Moreover, many clinical studies (see below) describing the immunophenotypic features of acute leukemias and correlating prognosis with immunophenotyping in ALL and AML have been performed with single-color analyses. It is obvious that these studies have not always been adequate to distinguish malignant from normal hematopoietic cells and, more important, have not made use of the information provided by multiparameter flow cytometry *(24)*. Although the ability of three- or four-color immunophenotyping to resolve

unique subsets of malignant cells reliably within a complex population has been convincingly demonstrated, and its application has substantially expanded our understanding of normal and malignant subsets of hematopoietic cells, the clinical relevance of this technique in classification of the acute leukemias has been demonstrated only for flow cytometric detection of MRD (reviewed in ref. *27*). Future studies in the acute leukemias will show whether the analysis of multivariate phenotypic patterns of leukemic blasts, including the density of antigen expression *(30,31)* and its pattern of reactivity (e.g., homogeneous vs heterogeneous), using well-established and hopefully standardized flow cytometric procedures, will provide additional diagnostically and clinically relevant information.

4. GENETIC CHARACTERIZATION

Genetic analysis is an obligatory diagnostic tool in acute leukemia. The results contribute to the confirmation of the diagnosis, but, more important, the karyotype of the leukemic blasts can give important information concerning the prognosis of the disease. Different methods can be used for the genetic characterization of leukemic blasts *(32)*. Chromosome banding analysis provides an overview of all chromosomal abnormalities that can be detected at the level of light microscopy. FISH, Southern blot analysis, or PCR can be used to detect submicroscopic mutations, although for these techniques a preknowledge of the genetic aberrations is mandatory for selecting the right probes.

4.1. Chromosome Banding Analysis

Using improved staining techniques, it had become possible by 1970 to identify individually each of the human chromosomes. This advance allowed the demonstration of recurrent cytogenetic abnormalities associated with specific subtypes of acute leukemia. Such abnormalities are often translocations, a rearrangement of two or more chromosomes that leads to the formation of a new fusion or chimeric chromosome. Also, deletion or amplification of genetic material may occur, consisting of the loss or gain of an entire chromosome or a portion of a chromosome.

Chromosome analysis requires fresh, viable leukemic blasts. Preferentially, bone marrow anticoagulated with heparin is used to study the acute leukemias. Fresh leukemic blasts are prepared directly or grown for 24–72 h in cell culture. Growing cells are then treated with colchicin, which arrests the cells in metaphase, a stage in cell division (mitosis) during which DNA is condensed into readily recognizable chromosomes. The cells are then swollen in a hypotonic solution, fixed, and dropped onto glass slides *(33)*. Different treatments are used to induce a characteristic banding pattern. To obtain a reliable result, a sufficient number of metaphases with good chromosome quality is required. According to an international convention, 20–25 metaphases are completely analyzed.

To interpret cytogenetic data, a nomenclature is needed to describe the karyotype. Currently, the International System for Human Cytogenetic Nomenclature, published in 1995, is used as a standard *(34)*. The normal diploid human cell contains 46 chromosomes, composed of 22 pairs of autosomes (numbered 1–22) and two sex chromosomes. Each chromosome has a constriction called a centromere that divides the chromosome into two arms. The short arm is designated p (for petit), and the long arm q. The arms of each chromosome contain a characteristic pattern of light and dark bands, and each band is identified by a number. The karyotype is reported as a list describing the number of chromosomes, the sex chromosomes present, and any observed abnormalities. Numeric and structural abnormalities can be distinguished. A gain of a complete chromosome is called trisomy and is denoted by a plus sign (e.g., 47,XX,+8), whereas the loss of a chromosome (monosomy) is indicated by a minus sign (e.g., 45,XY,–7). The most common structural abnormalities are translocations (t), deletions (del), inversions (inv), duplications (dup), and isochromosomes (i) *(34,35)*. The general rule in tumor cytogenetics is that only the clonal chromosomal abnormalities found in a tumor should be reported. This means that at least two cells with the same aberration were observed. If the abnormality is a missing chromosome, the same change must be present in at least three cells to be accepted as clonal *(34)*.

Chromosomal aberrations can be subdivided into those that are primary and those that are secondary. Primary aberrations are frequently found as the sole karyotypic abnormality, are often associated with a particular subtype of leukemia, frequently lead to specific gene rearrangements, and are believed to be essential in initiating leukemia. Secondary aberrations, on the other hand, are rarely or never found alone; they develop in cells already carrying a primary abnormality. They usually lead to genomic imbalances and are considered to be important in leukemia progression *(35–37)*.

4.2. Fluorescence In Situ Hybridization

The FISH technique is based on the hybridization of DNA probes that identify specific chromosomal structures. FISH needs suitable DNA probes for defined chromosomal regions known to be involved in karyotypic abnormalities. DNA within metaphase chromosomes or interphase nuclei is denaturated and then hybridized to a probe, which is either directly labeled with a fluorochrome or conjugated with a hapten that can be recognized by specific antibodies bearing a fluorescent tag. The corresponding chromosome structures can be visualized as fluorescent signals *(33)*. The kinds of probes used vary depending on the clinical setting and differential diagnosis. Probes derived from repetitive centromeric sequences, which recognize specific chromosomes, can be used to detect trisomies or monosomies, whereas probes specific for individual genes can be used to detect alterations in gene structure or number. Aberrations detected by such probes include deletions, inversions, translocations, and gene amplifications. Both kinds of probes can be applied on either intact interphase nuclei or metaphase chromosomes. Whole chromosomes can be stained (or "painted") with the collection of probes distributed along the full length of a specific chromosome. The probes can only be applied on metaphase chromosomes and can be used to characterize marker chromosomes of unknown origin. FISH is a helpful tool at diagnosis for the identification of numeric and structural abnormalities and can also be used to monitor effects of therapy. The detection of MRD is hampered by the low sensitivity of this technique.

FISH techniques have provided an increased ability to identify chromosome segments, to analyze and describe complex

rearrangements, and to detect genetic aberrations in interphase nuclei. These advances mandated the development of a FISH nomenclature, which was included for the first time in the International System for Human Cytogenetic Nomenclature in 1995 *(34)*. FISH results can be included in the karyotype description obtained by chromosome banding analysis. If FISH results are obtained on metaphases, the abbreviation *ish* is used. If FISH is carried out on interphase nuclei, the term *nuc ish* is used. The chromosome bands as well as the loci of the probes used are named. The gains and losses of chromosomal material are indicated by + and –; the copy numbers of distinct loci are given by a multiplication sign and the copy number. The juxtaposition of probes is indicated by the abbreviation *con*. For example, the interphase FISH result in cells carrying a *BCR-ABL* rearrangement owing to the translocation t(9;22)(q34;q11) is reported as nuc ish 9q34(ABLx2), 22q11(*BCR*x2)(ABL con *BCR*x1). If probes that are normally juxtaposed are separated owing to an rearrangement, the abbreviation *sep* is used.

In 1996, two methods were published that are able to identify each of the 24 different human chromosomes (22 autosomes and the two sex chromosomes, X and Y) with uniquely distinctive colors *(38–40)*. The new techniques complement standard cytogenetics and are especially helpful in deciphering complex or hidden chromosomal rearrangements *(38,41)*.

4.3. Comparative Genomic Hybridization

If no viable cells are available or cells do not proliferate in vitro, chromosome analysis cannot be performed. Recently, an alternative approach was introduced that does not rely on tumor metaphases. This technique, called comparative genomic hybridization (CGH), provides an overview of DNA sequences and copy number changes (losses, deletions, gains, amplifications) in a tumor specimen and maps theses changes on normal chromosomes *(42,43)*. CGH is based on the *in situ* hybridization of differentially labeled total genomic tumor DNA and normal reference DNA to normal human metaphase chromosomes. Copy number variations among the different sequences in the tumor DNA are detected by measuring the tumor/normal fluorescence intensity ratio for each locus in the normal metaphase chromosomes. CGH only detects changes that are present in a substantial proportion of tumor cells (>50%). It does not reveal translocations, inversions, and other aberrations that do not change copy number. In leukemia, this method is only helpful in cases in which no metaphases of the leukemic cell clone can be obtained or if it is suspected that only the normal hematopoietic cells, not the leukemic blasts, divide in vitro. In ALL, this technique seems to add more information to chromosome banding analysis than it does in AML *(44–48)*.

4.4. Polymerase Chain Reaction

The PCR technique permits in vitro production of many copies of a defined DNA sequence up to several kilobases long, provided DNA sequences flanking this region on both sides are known *(49)*. The amplified DNA fragment can be detected as a band after gel electrophoresis. PCR can detect translocations, inversions, insertions, deletions, duplications, point mutations, and amplifications. Its advantage and disadvantage are one and the same: its extreme sensitivity. The technique can detect as few as 1 leukemic cell in 10^4–10^6 normal cells and therefore is very useful for the detection of MRD after cytoreductive therapy. A problem is the risk of false-positive results owing to contamination of reagents. New opportunities are emerging with the development of quantitative PCR. With techniques called real-time PCR, quantification of PCR products is more easily and more accurately reached than before, allowing one to determine the kinetics of leukemic cells during and after chemotherapy.

4.5. DNA Microarray Technology

DNA microarray technology allows one to assay thousands of unique nucleic acid samples simultaneously. This technology consists of the immobilization or synthesis of nucleic acids at high density on a solid support. The array is used as a detector for the hybridization of a complementary, fluorescently labeled nucleic acid probe *(11,12)*. Gene expression profiles can be determined, and sequence analysis of polymorphisms and detection of mutations can be performed with this new technique. In contrast to conventional methods that are limited to the analysis of expression of a few genes, the DNA array technology is capable of analyzing the profile of all genes expressed in a cell or organ. The first experiments on cell lines established from leukemia have proved that this new technique is reproducible and allows the analysis of downstream genes of leukemia-specific fusion transcripts. Golub et al. analyzed 27 patients with ALL and 11 with AML and were able to define 50 of 1100 genes that allow ALL to be distinguished from AML *(13)*. Gene expression profiling with the help of DNA microarrays might add useful information for the classification of acute leukemias and, more important, will lend insight into the pathogenetic mechanisms of leukemia in the future.

5. FAB-CLASSIFICATION OF AML

The FAB classification of AML follows an algorithm and distinguishes 11 different subtypes *(3–6,18,19)*. These strict definitions of AML do not take into account underlying biologic characteristics *per se*. Without the FAB categories, however, chromosomally defined entities and most correlations between morphology and cytogenetics would have been missed in the last 25 years. Moreover, correlations between diagnostic and therapeutic results in AML were first recognized by use of the FAB system. This is still the case today.

5.1. Correlations of Morphologic and Cytochemical Features with Immunophenotyping, Cytogenetics, and Molecular Genetics

Since publication of the original FAB classification for acute leukemias in 1976, several revisions have appeared. Although the concordance rate among observers differed from 65% to 80% *(3,50–52)*, it was possible to correlate specific and recurrent morphologic features with cytogenetic and immunophenotypic results. In AML, use of the FAB system led to the description of more specific morphologic details and correlations with cytogenetic results (Table 1):

1. AML M2 (or-M1), showing dysgranulopoiesis, increase of normal eosinophils, very mature blasts (type II and type III), and long, needle-like Auer rods were found to be associated cytogenetically with t(8;21) *(53,54,55,56)*.

Table 1
FAB Classification of AML

Subtype	*Definition*
AML-M0	No maturation, MPO <3%, *but* myeloid markers by immunophenotyping present
AML-M1	Blasts ≥ 90% of nonerythroid cells, MPO ≥ 3%
AML-M2	>10% of myeloid cells show maturation from promyelocytes onwards, monocytes <20%
AML-M3	Predominant cells are highly abnormal promyelocytes
AML-M3v	Predominant cells are bilobulated blasts with strong MPO reactivity
AML-M4	Myelomonocytic blast cells, with monocytic component >20% but <80%
AML-M4eo	Like M4, with abnormal eosinophils (usually >5%)
AML-M5a	Monoblasts ≥ 80% in bone marrow
AML-M5b	Monoblasts and monocytes ≥ 80% in bone marrow
AML-M6	Erythroblasts ≥ 50% of total nucleated cells, *and* ≥ 30% of nonerythroid cells are blasts
AML-M7	Blasts demonstrated to be megakaryoblasts by immunophenotyping (CD41+, CD61+)

Abbreviations: FAB, French–American–British; MPO, myeloperoxidase.

2. AML M2 baso showed an increase of basophils besides the typical M2 morphology and was in some cases correlated with t(6;9) *(57)*.
3. AML M2, with increased megakaryocytes, mostly with two little nuclei, and in many cases normal or even elevated platelet counts, was seen in cases with inv(3)(q21q26) *(58,59,60)*.
4. AML M3 *(61)* and its variant (AML M3v) *(19)* were found to bear the same cytogenetic abnormality, namely, t(15;17).
5. In AML M4, with abnormal eosinophils (M4eo), inv(16) or t(16;16) was detected *(62–65)*. In contrast to normal eosinophils, these abnormal eosinophils show large basophilic granules and abnormal granular positivity with CAE.
6. In cases of AML M4 or AML M5 with erythrophagocytosis, t(8;16) was found.
7. Another correlation was observed in cases with dysplastic granulopoiesis and pseudo-Pelger-Huët anomalies, sometimes showing alterations in chromosome 17p involving the *p53* gene *(66)*.
8. In cases of monocytic AML (M4, M5), the 11q23 chromosome region was frequently involved.

The strongest correlations between cytomorphology and cytogenetics were clearly found in AML M3(v) and AML M4eo; all other associations are much weaker. It should be noted, however, that only cytogenetic and/or molecular genetic analyses can lead to the definition of a biologic entity. For example, the threshold of 5% abnormal eosinophils for the diagnosis of AML M4eo is arbitrary, because some cases show < 1% clearly abnormal eosinophils.

5.2. Clinical Impact of Specific Morphologic Features on Diagnosis

In AML, but not ALL, more detailed morphologic approaches have led to deeper insights into the biology of acute leukemias and allow one to predict the prognosis more precisely. The presence of dysplasia, for example, is now included in the new WHO proposal *(20)*. It therefore seems appropriate to present some data from the literature as well as our own data [from the AML Cooperative Group (AMLCG)].

5.2.1. Auer Rods

The detection of Auer rods can lead to the diagnosis of AML. However, the AMLCG study detected Auer rods in only 45.5% of 601 cases of AML *(67)*. It is well known that Auer rods may give important information in AML, as follows *(4,16,68,69)*:

1. In cases with MPO < 3% (normally classified as AML M0), the detection of Auer rods alone leads to the diagnosis of AML M1, by definition.
2. In AML M1 and-M2 carrying t(8;21), very often long, thin, so-called needle-like Auer rods can be seen, and one may even predict this aberration before the cytogenetic result is available.
3. In most cases of AML M3 (and more or less in M3v), bundles of Auer rods can be seen in the cytoplasm. These cells are called faggot cells. However, only the detection of t(15;17) or the *PML/RARα* fusion gene will make the diagnosis absolutely safe.

In pediatric AML studies, the detection of Auer rods was correlated with a better prognosis *(70,71)*. In our study of 601 adult patients with AML, the detection of Auer rods was correlated with a better prognosis with respect to overall survival ($p = 0.0001$), relapse-free survival ($p = 0.01$), and event-free survival ($p = 0.0003$). This was also true when patients with t(8;21) or t(15;17) were excluded from the cohort of Auer rod-positive patients *(67)*.

5.2.2. Dysplastic Features at Diagnosis

The detection of dysplasia was described as essential in myelodysplastic syndromes but was also investigated in *de novo* AML at diagnosis *(67,72–83)* and during remission *(74)*. However, studies in AML were mostly retrospective, and criteria for the definition of dysplasia were not generally accepted. This makes it very difficult to compare results. The WHO classification referred to this morphologic feature and defined an AML subgroup with multilineage dysplasia as the presence of dysplastic features in two or more cell lines *(20)*. In our investigations, we followed the definitions of Goasguen and Bennett *(76)*:

1. *Dysgranulopoiesis (DysG)* was defined as agranular or hypogranular features, or hyposegmented nuclei (pseudo Pelger-Huët anomaly), in >50% of at least 10 PMNs. At least 25 cells were observed, but usually 100 cells were counted. MPO deficiency in the PMNs was defined as >50% MPO-negative cells in at least 10 PMNs after strong positivity of eosinophils or other PMNs was confirmed.
2. *Dyserythropoiesis (DysE)* was defined as dysplastic features in >50% of at least 25 erythroid precursors, including megaloblastoid aspects, karyorrhexis, nuclear particles, or multinuclearity. A minimum of 25 cells have to be counted.

Table 2
Relationships Among Morphology, Immunophenotypic Features, and Genetic Aberrations in AML[a]

Antigen	*M0*	*M2t(8;21)*	*M3t(15;17)*	*M4Eoinv(16)*	*M5*	*M5t(9;11)*	*M7*
MPO	+/–	+	+	+	–/+	–	–
CD2	–			+/–			
CD7	–/+	–	–	–	–/+	–	–/+
CD13	+/–	+	+	+	+/–	–/+	+/–
CD14	–	–	–	+/–	+/–	–/+	–
CD15	–	+/–	–/+	+/–		+	–
CD19	–	+/–					
CD33	+/–	+/–	+	+	+	+	+/–
CD34	+/–	+/–	–	–/+			
CD56		+/–				+/–	
CD41/ CD61	–	–	–	–	–	–	+
CD64	–	–	+/–	+	+	+/–	
CD65	–/+	+/–	–/+	+	+/–	+	+/–
CD117	+/–	+/–	–/+	+/–	–/+		
HLA-DR	+/–	+	–	+	+	+	+/–

[a] –, antigen not expressed; –/+, antigen expressed in <50% of patients; +/–, antigen expressed in the majority of patients; +, antigen expressed; open fields represent partial expression without specificity for diagnosis or lack of reliable data.

3. Dysmegakaryopoiesis (DysM) was diagnosed when at least three megakaryocytes or >50% of at least six cells showed dysplastic features, such as microkaryocytes or multiple separated nuclei or very large single nuclei.

Trilineage dysplasia (TLD) was diagnosed when DysG, DysE, and DysM were detectable. In the AMLCG series of 601 prospectively analyzed patients with *de novo* AML, no dysplasia was seen in 45.1%, DysG was seen in 16.7%, DysE in 10.2%, DysM in 22.3%, and trilineage dysplasia in 14.3%. Although there was a trend toward better overall survival in patients without any dysplasia in comparison with multilineage dysplasia (i.e., two cell lines) or TLD, none of the differences tested were statistically significant in this cohort *(67)*.

The analysis was also not able to demonstrate significant differences between patients with no dysplasia and one-lineage dysplasia (combined group) vs patients with multilineage dysplasia, after we excluded all patients with specific cytogenetic abnormalities according to the definition of the WHO proposal *(20)*.

Another cohort was defined for subgroup analysis: we investigated only patients with normal karyotype with respect to dysplastic features. Patients with no dysplasia or single-lineage dysplasia showed no significant difference from patients with multilineage dysplasia in relapse-free ($p = 0.4$) or event-free survival ($p = 0.1$). These results stress the need for further investigations. Subgroup analysis has to be performed before multilineage dysplasia in AML can be proved to define an independent biologic entity.

5.2.3. Dysplasia and Its Correlation with Secondary AML

In many studies, the detection of dysplasia in one to three cell lineages has been used to define a case as *secondary* AML after an antecedent myelodysplasia. There is no clinical evidence that this conclusion is true *(79)*. The discrimination between *de novo* AML and AML after a myelodysplastic syndrome (MDS) can be made only from the history of the patient (i.e., abnormal white blood cells and unexplained anemia or thrombocytopenia in the past). If this is not possible, and the patient´s history with respect to fatigue or other adverse features lasts only some weeks or up to 2–3 mo, one cannot predict that any AML with specific dysplastic features evolved from a preceding MDS. Drawing conclusions like this does not improve understanding of AML pathogenesis and may even lead to wrong treatment decisions.

6. IMMUNOPHENOTYPING OF AML

Immunophenotyping by flow cytometry has been instrumental in recognizing minimally differentiated AML (AML M0), acute megakaryoblastic leukemia (AML M7), and AML coexpressing lymphoid-associated antigens *(5,6,25,28)*. It has been especially helpful in distinguishing AML with monocytic differentiation from AML M0/M1 or AML subtypes with granulocytic differentiation (i.e., AML M2/M3) *(84)*. The diagnostic sensitivity of a comprehensive panel of MAbs to myeloid and lymphoid lineage as well as progenitor cell-associated antigens has been demonstrated in both childhood and adult AML *(25,85–88)*. Although none of the antimyeloid MAbs used in these studies recognized the blast cells of all AML patients, nearly all AML cases can nowadays be detected by using a combination of two or three panmyeloid reagents (i.e., CD13, CD33, CD65) with MAbs to MPO that detect both the proenzymatic and the enzymatic forms of MPO. Except for MPO and megakaryocyte-associated antigens (e.g., CD41a, CD61), however, expression of myeloid-associated markers is not restricted to AML. Attempts to correlate immunophenotypic features with the various AML subtypes (AML M1 through AML M6) according to the FAB classification have been largely unsuccessful (22,25,89). Although some AML subtypes (e.g., AML M3; see below) show a characteristic immunophenotypic profile, there are few entirely consistent relationships between morphology and immunophenotype (Table 2). Thus, cases with identical antigen expression may belong to different FAB subtypes, and different immunophenotypic features are found in the same FAB subtypes.

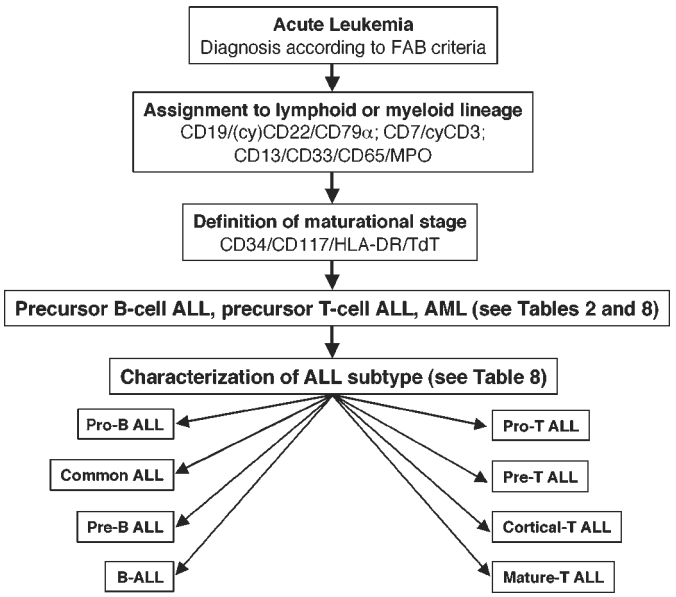

Fig. 1. Flow chart indicating the antigens essential for lineage assignment and definition of maturational stage in AML and ALL.

In AML, interpretation of immunophenotyping studies may be confusing, because leukemic blasts in bone marrow and peripheral blood specimens are frequently admixed with normal hematopoietic cells, and the blast cell population can also be heterogeneous. Therefore, various multiparameter flow cytometry techniques have been proposed that facilitate the identification of leukemic cells. Among these techniques, the leukocyte common antigen (CD45)/side-scatter (SSC) gating procedure allows an efficient discrimination between the blast cell population and the normal cells and facilitates the analysis of leukemic blasts present in low proportions (90,91). The use of CD45/SSC gating, primarily gated on blast cells identified by virtue of low/intermediate CD45 density, correlates with bone marrow differential and provides characteristic flow-cytometric profiles for most subtypes of AML *(25,84,91)*. Moreover, this gating strategy has been demonstrated to give similar results in leukemic specimens enriched for leukemic blasts by density-gradient separation techniques and in lysed whole bone marrow or peripheral blood samples *(91)*. Based on these observations, several authors suggested that CD45/SSC gating should replace forward scatter (FSC)/SSC gating, and that this method could contribute to reduced costs without affecting diagnostic quality *(91–93)*.

By applying multiparameter flow cytometry to immunophenotyping studies, it has recently been shown that the antigenic profiles of AML differ significantly from the antigen expression pattern on normal bone marrow elements. These phenotypic aberrations of leukemic blasts, often referred to as asychronous or aberrant antigen expression, are probably related to the underlying genetic alterations as well as to a disturbed regulatory control of particular proteins *(94–96)* and may be useful in both screening for genetic abnormalities and monitoring of MRD.

Several antigen-screening panels for immunophenotyping of AML have been recommended *(25,28,97,98)*, mainly including MAbs directed toward antigens expressed by early hematopoietic progenitors and relatively lineage-restricted antigens. The screening panel currently applied to immunophenotypic characterization of acute leukemias in our institution is presented in Fig. 1.

In the following sections, we describe the immunophenotypic features of AML M0 and AML M7 and summarize the antigenic profiles that may be associated with clinically relevant entities such as AML with t(8;21), acute promyelocytic leukemia with t(15;17), AML with abnormal bone marrow eosinophilia and inv(16) or t(16;16), and AML with 11q23 abnormalities (Table 2). Although the relevance of immunophenotyping for the identification of AML subtypes carrying a specific genetic abnormality has been questioned, recent studies based on multivariate phenotypic pattern and light scatter characteristics instead of individual antigen expression have demonstrated markedly improved sensitivity and specificity of immunophenotyping (94,99).

6.1. AML Minimally Differentiated (*AML M0*)

Leukemias of the M0 subtype, which cannot be recognized on morphologic grounds alone, comprise 3–6% of pediatric and up to 10% of adult AML cases. In 1991, the FAB Cooperative Group listed morphologic, cytochemical, and immunophenotypic diagnostic criteria and proposed the designation M0 for these leukemias *(6)*. These criteria included negative cytochemical reactions to MPO and SBB stains, no evidence of lymphoid differentiation by immunophenotyping, and expression of myeloid antigens (e.g., CD13 or CD33) or the demonstration of the enzyme MPO by immunophenotyping and/or electron microscopy.

More recently, stricter guidelines for excluding lymphoblastic and megakaryoblastic leukemias have been proposed. They are based on the availability of more specific lineage-restricted MAbs, the use of multicolor flow cytometry, and the cytoplasmic detection of myeloid antigens in fixed cells (e.g., CD13, MPO) *(25,28,100)*. According to these criteria, acute leukemias devoid of detectable MPO can only be classified as AML M0 in the absence of lineage-restricted lymphoid (e.g., CD3, CD22, CD79α, TCRβ) and megakaryocytic antigens (e.g., CD41, CD61). Most M0 cases express CD13, CD33, and CD65 as well as progenitor cell-associated antigens, such as HLA-DR, CD7, CD34, and CD117 *(101–104)*, whereas other myeloid lineage-associated antigens (e.g., CD14, CD15) are rarely found. The reliability of anti-MPO antibodies for detecting minimal myeloid differentiation in cases with CD13 and CD33 negativity has been demonstrated *(102,105,106)*. Up to 80% of M0 cases may have a complex composite immunophenotype with expression of myeloid as well as non-lineage-restricted lymphoid markers, including CD2, CD7, CD4, CD19, and terminal deoxynucleotidyl transferase, which makes them difficult to classify as AML, ALL, or biphenotypic acute leukemia (see below) *(100)*. Cytogenetic studies reveal a variety of clonal abnormalities (such as complex karyotypes, anomalies of chromosome 5 and/or 7, trisomy 8, and trisomy 13, reflecting the heterogeneity of minimally differentiated AML) and indicate that AML M0, very similar to AML M1, is not a unique leukemia subtype but probably includes distinct malig-

nant myeloid processes with different underlying cytogenetic and/or molecular genetic defects *(101,102)*. The cytogenetic abnormalities and the higher level of P-glycoprotein expression described in most but not all studies may contribute to the poor treatment outcome that has been observed in adults with AML M0 *(102,103,107)*.

6.2. AML M2 Harboring t(8;21)

Several studies have described the distinctive immunophenotypic features of AML M2 cases harboring the translocation t(8;21), which complement the characteristic morphologic findings of this AML subtype *(96,108–111)*. These features include expression of CD13, CD15, CD33, CD34, CD65, and HLA-DR with frequent coexpression of the B-cell-associated antigen CD19 and the neural cell adhesion molecule CD56. Notably, however, recent case reports of adult patients with a so-called myeloid surface antigen-negative phenotype have been published *(112–114)*, indicating that low levels or absence of the panmyeloid antigens CD13 and CD33 may occur in parallel with expression of MPO as detected by cytochemical staining or flow cytometry. Controversial findings have been described regarding the frequency and intensity of CD19 and/or CD56 expression in AML M2 with t(8;21) *(87,115)* and have led to questions as to whether these aberrant phenotypic features occur frequently enough to allow selection of cases for molecular screening on the basis of immunophenotyping *(110)*. This discrepancy may be caused by the usually weak and variable expression of CD19 and CD56 on AML cells, thus requiring special gating strategies to separate blasts from whole mononuclear cell fractions in flow-cytometric analysis and by other methodologic aspects, including the use of different CD19 MAbs and the application of different staining techniques. Recent studies have shown overexpression of CD34 in an asynchronous combination with cytoplasmic MPO and have suggested that quantification of CD34 expression might be useful for both rapid diagnosis and remission assessment in AML with t(8;21) *(96)*.

6.3. Acute Promyelocytic Leukemia with t(15;17)

The characteristic but not unique immunophenotypic features of acute promyelocytic leukemia APL with the t(15;17) include (1) absence or low expression of HLA-DR, CD7, CD14, CD15 (as detected with the MAbs VIM-D5 or VIM-C6), and CD34; (2) variable expression of CD11b, CD65, and CD117; and (3) weak expression of CD64 and strong expression of CD9, CD13, CD33, CD68, and MPO *(84,87,116–118)*. More recently, the combined use of three phenotypic criteria [i.e., presence of a single blast cell population, heterogeneous reactivity for CD13 and the pattern of expression of CD34/CD15 as detected by the Leu-M1 MAb] *(94)*, and the availability of antibody reagents directed against the promyelocytic leukemia (PML) protein *(119)* have improved the sensitivity and specificity of immunophenotyping studies for rapid screening of APL, especially in cases with M3v morphology or rare cases of t(15;17)-positive leukemias resembling AML M1 and AML M2 *(120,121)*. In addition, immunophenotyping has been helpful in differentiating acute myelomonocytic or monocytic leukemias from the microgranular variant, which usually, unlike AML M4 or-M5, does not express CD4, CD14, CD36, and HLA-DR and exhibits distinct light-scatter characteristics owing to its high MPO content. Recent studies suggested a correlation between immunophenotypic characteristics and morphologic, molecular-genetic, and clinical features of childhood and adult APL that may be useful for a better determination of the biologic and clinical heterogeneity of this subtype. A strong association of the S (short) transcript resulting from a break at *bcr3* of the *PML* gene and M3v morphology with CD2 positivity has been described *(118,122,123)*, as well as a more frequent expression of CD56 in APL with the S-isoform subtype *(124)*. Interestingly, both aberrant immunophenotypic features were of prognostic significance, with CD2 positivity predicting a better complete response (CR) rate and event-free survival in APL *(118)*, whereas CD56 was associated with a poor treatment outcome in a small series of adult patients treated with various treatment protocols *(124)*.

Immunophenotypic features of rare AML cases, which morphologically resemble AML M3 and do not express the *PML-RARα* fusion gene but show rearrangement of the *RARα* locus with genes other than *PML* on chromosome 15, such as t(11;17) and t(5;17), are similar to the pattern seen in typical AML M3. Interestingly, expression of CD56 in parallel with functional natural killer (NK) cell-mediated cytotoxicity was observed in four cases with t(11;17) *(125,126)*.

6.4. *AML M4Eo*

The diagnostic value of multiparameter flow cytometric analysis for the detection of small subpopulations with aberrant immunophenotypic features has been nicely illustrated in AML M4Eo by demonstrating expression of CD2 on leukemic blasts with inv(16) or t(16;16) *(127,128)*. By using multiparameter flow cytometry, two major leukemic cell populations are evident in AML M4Eo with expression of panmyeloid and granulocytic or monocytic antigens, including CD4, CD13, CD14, CD15, CD33, and CD65. Similar to AML M2 with t(8;21), other characteristic features of AML M4Eo include frequent positivity of CD34 and the absence of CD7 *(87)*. The availability of MAbs to the chimeric CBFβ-MYH11 protein may be used in flow cytometric analyses to screen for the inv(16) abnormality *(129)*.

6.5. *AML M5* with 11q23 Aberrations

Previous studies in patients with acute myelomonocytic leukemias associated with 11q23 aberrations failed to show a specific immunophenotypic pattern on leukemic blasts that might be used to distinguish acute myelomonocytic leukemias with 11q23 translocations from FAB M4 or M5 cases without 11q23 involvement *(130,131)*. However, results in children and adults with *MLL* rearrangements, usually owing to t(9;11), disclosed characteristic features such as strong expression of HLA-DR, CD33, CD65, and CD4, whereas other myeloid lineage-associated antigens (e.g., CD13, CD14) and CD34 were detected in <30% of cases *(87,132,133)*. Furthermore, we *(133)* and others *(134)* have observed a frequent coexpression of CD56 in AML with monocytic differentiation and rearrangement of the *MLL* gene.

Interestingly, previous studies testing the reactivity of a MAb, 7.1, which detects the human homolog of the rat NG2 chondroitin sulfate proteoglycan molecule, have noted strong associations among blast cell expression of the NG2 molecule, FAB M4/M5 morphology, and 11q23 abnormalities in child-

hood AML *(135,136)*. In agreement with these results, we recently demonstrated in a large series of patients, including children and adults with AML, that the MAb 7.1 is a sensitive but not entirely specific marker for the identification of 11q23-associated AML *(133)*. Moreover, we observed frequent coexpression of NG2 and CD56 in AML with monocytic differentiation, raising the question of whether these molecules, both probably involved in cell adhesion and migration mechanisms, have any pathophysiologic impact on the clinical behavior of this AML subset.

Recent studies suggested that addition of CD64 and CD45 intensity vs logarithmic side-scatter analysis to CD14, a highly specific but relatively insensitive monocytic marker, may greatly improve flow cytometric detection of AML with monocytic differentiation *(84)*. In addition, data from a large series of Japanese adult patients suggested that AML with myelomonocytic differentiation, often associated with 11q23 abnormalities or with inv(16), exhibited a typical surface antigen expression pattern (i.e., $CD34^{low}$, $CD33^{high}$, $CD11b^{high}$, GM-CSF-R^{high}, and $CD4^{positive}$) *(137)*.

In future studies, it will be important to determine whether the analysis of these phenotypic features by multiparameter flow cytometry will contribute to a more relevant subdivision of AML with monocytic differentiation and whether such studies have prognostic implications.

6.6. Acute Megakaryocytic Leukemia (AML M7)

The differentiation of AML M7 from ALL, AML M0, and sometimes small tumors in children is usually not possible by morphologic and cytochemical studies. Therefore, the diagnosis of AML M7 must be confirmed by immunophenotypic detection of different platelet glycoproteins indicating megakaryocytic differentiation (e.g., CD41a, CD61) or by ultrastructural demonstration of platelet peroxidase *(5,18)*. Immunophenotyping studies, however, are more readily performed than ultrastructural studies and have largely replaced the latter *(138,139)*. Leukemic blasts in AML M7 express CD61, CD41a, and (less frequently) CD42b. In addition, most cases express CD4, CD33, CD34, CD36, HLA-DR, and (less frequently) CD13. Coexpression of lymphoid antigens, especially CD7 or CD2, has been described. Cytoplasmic expression of platelet glycoproteins may precede the cell surface expression of these markers and should be tested in cases with undifferentiated morphology and negative or inconclusive cytochemistry to differentiate AML M7 from AML M0 and ALL *(140)*. Caution must be exercised, as platelet adherence to leukemic blasts as well as nonspecific binding of glycoproteins IIb/IIIa to AML M5 may result in false-positive CD41a and CD61 staining results. Therefore, confirmation of flow cytometric results by cytospin immunofluorescence should be performed in all cases with equivocal immunophenotyping and morphologic findings.

Recently, characteristic immunophenotypic features have been described within the CD34+ stem-cell compartment in patients with AML M7 *(141)*. In comparison with CD34+ cells in AML M0 through AML M6 subtypes, the CD34+ megakaryoblasts expressed CD61 and glycophorin A but were CD38–. These results are in line with the hypothesis of a common immature progenitor cell for the megakaryocytic and erythroid cell lineages *(142)* and the expression of megakaryocytic antigens occasionally observed in acute erythroleukemia as well as the positivity of glycophorin A in some cases of AML M7.

The chromosomal abnormalities associated with AML M7 include t(1;22)(p13;q13) *(143)*, constitutional or acquired trisomy of chromosome 21 *(144–147)*, and occasionally rearrangements of 3q21 and 3q26. In contrast to t(1;22), which is exclusively observed in infants and is not associated with any dysplastic features, rearrangements of 3q21 and 3q26 are not specific for AML M7, have been demonstrated in all subtypes of AML except AML M3, and occur mainly in older patients whose leukemic blasts may display dysplastic features *(60)*.

It should be noted that infants with Down's syndrome often present with a transient myeloproliferative disorder, which, by immunophenotyping studies, commonly shows evidence of megakaryocytic as well as erythroid differentiation and by light microscopy or immunophenotyping is indistinguishable from AML *(146,147)*. Leukemic blasts in children with Down's syndrome and AML M7 often show evidence of erythroid differentiation as well and coexpress CD7 *(146,147)*.

6.7. Prognostic Implications of Immunophenotyping in AML

The prognostic significance of surface antigen expression in AML is still a matter of controversy. Although some investigators, especially in childhood AML, could not show any correlation between the expression of individual progenitor-, myeloid- or lymphoid-associated antigens and treatment outcome *(87,148,149)*, others suggested a significant influence of specific antigens or combined phenotypic features on the CR rate and/or CR duration and survival. Among the antigens implicated as having an adverse prognostic effect are CD7, CD9, CD11b, CD13, CD14, HLA-DR, CD34, and TdT *(106,150–153)*. On the other hand, the presence of CD15, CD65, and CD2 has been associated with a better treatment outcome *(154,155)*. Other authors could not confirm these findings (e.g., CD2, CD7, CD34, TdT) *(87,148,149,155–157)*. The comparability of most of these results, however, is hampered by methodologic differences such as the choice of MAbs and techniques applied to the detection of antigen expression, inconsistencies in criteria for defining antigen positivity, and variation in the patient populations studied (i.e., children and/or adults) or the treatment administered.

Moreover, the prognostic value of correlating clinical outcome with specific antigens rather than evaluating the composite immunophenotype must be questioned in view of recent findings demonstrating that expression of particular antigens can be associated with favorable as well as poor prognostic genetic aberrations. For example, t(8;21), inv(16), chromosome 5 and 7 aberrations, and complex karyotypes were more frequently observed in CD34+ AML *(86,158)*, and CD19 coexpression may occur in AML with either t(8;21) or t(9;22) *(87,159,160)*. These results suggest that CD34+ and/or CD19+ AML comprise a heterogeneous group of patients with good as well as poor risk factors. Recent data suggesting a prognostic role of CD56 expression in AML with t(8;21) *(111)*

and APL *(124)* but not in AML with 11q23 translocations *(130)* are in line with this statement. Moreover, in future studies, expression of surface antigens should also be interpreted in the context of other cell biologic features, including differentiation stage and functional characteristics reflecting cellular resistance mechanisms to cytotoxic drugs (e.g., multidrug-resistance phenotype, expression of apoptosis-regulating proteins) *(157,158,161–163)*. Our own results in a large series of untreated children and adults with *de novo* AML enrolled in the German AML-Berlin-Frankfurt-Münster (BFM) and AMLCG studies do not show any influence of the expression of individual myeloid-, lymphoid-, and progenitor-cell-associated antigens on prognosis *(87,156,164,165)* and thus do not indicate that immunophenotyping alone can be applied in risk stratification in AML at diagnosis. These findings are in line with other recent studies in children *(148,149)* and adults *(85)* with AML.

7. GENETIC ABNORMALITIES IN AML

Cytogenetic analysis is the most important diagnostic tool for determining prognosis in AML *(166–169)*. Cytogenetic studies have revealed that acquired clonal chromosome aberrations can be observed in most patients with AML *(35)*. Numerous recurrent karyotypic abnormalities have been discovered in AML (Table 3) *(37,170)*. Chromosome analysis has paved the way for molecular studies that have identified genes involved in the process of leukemogenesis *(171)*. Furthermore, the identification of specific chromosomal abnormalities and their correlation with cytomorphologic features, immunophenotype, and clinical outcome have led to a new understanding of AML as a heterogeneous group of distinct biologic entities. The importance of cytogenetic findings in AML for classification and for the understanding of pathogenetic mechanisms is increasingly appreciated in a clinical context and also in the new WHO classification, which uses cytogenetic abnormalities as a major criterion *(20)*.

The incidence of abnormal karyotypes in AML has been reported to be 55–78% in adults and 77–85% in children *(35,172–178)*. However, a substantial proportion of patients with AML have no chromosome abnormalities. Although it is possible that normal karyotypes may be attributed in some cases to the existence of nonmalignant cells dividing preferentially in vitro, the fact that in many patients the normal karyotype observed at diagnosis remains normal at relapse suggests that the absence of cytogenetic aberrations is a real phenomenon rather than a failure to detect aberrations *(179,180)*. Recent data indicate that a proportion of cytogenetically normal patients displays submicroscopic gene alterations that can only be detected by molecular methods. For instance, approx 6% of adult AML patients with a normal karyotype display a partial tandem duplication within the *MLL* gene *(181,182)*.

Attempts to classify cytogenetic data in AML have led to recognition of two distinct karyotypic patterns. One is characterized by balanced rearrangements leading to specific gene rearrangements, whereas in the other, unbalanced aberrations result in large-scale genomic imbalances. According to the hypothesis of Johansson et al. *(36)*, there are no unbalanced primary aberrations. An unbalanced "primary" abnormality is secondary to a submicroscopic, truly primary change. Therefore, in cases without a balanced primary abnormality, molecular analysis might reveal the underlying primary defect. Especially in patients with complex aberrant karyotypes, who show a variety of different unbalanced aberrations, submicroscopic abnormalities (such as mutations in DNA repair genes) leading to genetic instability must be suspected.

Table 3
Chromosomal Abnormalities in AML

			Frequency	
Cytogenetic change	*Fusion gene*	*FAB subtype*	*Children (%)*	*Adult (%)*
t(8;21)(q22;q22)	*AML1-ETO*	M2/M1	10–15	8–12
inv(16)(p13q22)	*CBFβ-MYH11*	M4eo	6–12	8–12
t(15;17)(q22;q12)	*PML-RARα*	M3/M3v	8–15	8–10
t(9;11)(p22;q23)	*MLL-AF9*	M5a	8–10	1–2
t(3;21)(q26;q22)	*AML1-EAP/EVI1*	—	1	<1
t(6;9)(p23;q34)	*DEK-CAN*	M1/M2	1–2	Rare
inv(3)(q21q26)	*EVI-?*[a]	—	<1	1–2
t(1;22)(p13;q13)		M7	2	–
+8 sole		—	1–4	3–5
+11 sole		M1/M2	–	<1
Complex		—	6	10–20

[a]?, unknown.

7.1. Primary Chromosome Abnormalities and Their Molecular Correlates

Primary chromosomal aberrations are frequently found as the sole karyotypic abnormality and are often specifically associated with a particular AML subtype. On average, 55% of AML patients with karyotypic abnormalities have only one rearrangement (15–20% have gain or loss of a single chromosome) *(35)*.

7.1.1. t(8;21)(q22;q22)/*AML1-ETO*

A t(8;21)(q22;q22) was first identified in 1973 *(183)*. This is the most frequent abnormality in AML in children (incidence, 10–15%) and occurs in approx 7–10% of patients with AML in Europe and the United States *(35,184)*. However, its frequency varies, and it is reported to be particularly common in Japan (37.5%) and in South Africa (62.5%) *(35)*. The 8;21-translocation is more frequent in the young and is rare beyond the age of 50. In >90% of patients, it is associated with a FAB-M2 subtype (around 10% show a M1-subtype) *(55,185)*. The translocation breakpoints have recently been cloned *(186,187)*. The breakpoints in 21q22 cluster to a limited region of the *AML1* gene, which is an important transcription factor in hematopoietic cells *(188,189)*. The 8q22 breaks cluster to the locus of a gene with putative zinc finger DNA binding motifs called *ETO* or *MTG8*. The translocation leads to a consistent hybrid gene encoding a novel message that can be consistently detected by reverse transcriptase RT-PCR *(190)*. Data on the detection of MRD with RT-PCR demonstrate that even in patients in long-term clinical remission, *AML1-ETO* fusion transcripts are still detectable *(191)*. New methods allowing the quantification of transcripts may be more helpful for treatment decisions because the kinetics of the amount of transcripts may be more important than the observation that *AML1-ETO* transcripts are still detectable *(192)*.

To elucidate the role of *AML1-ETO* in leukemogenesis, mice with a knocked-in *AML1-ETO* gene were generated *(193)*. These studies suggest that *AML1-ETO* not only neutralizes the normal biologic activity of *AML1* but also directly induces aberrant hematopoietic cell proliferation *(194)*.

7.1.2. t(15;17)(q22;q12)/*PML-RAR*α

The t(15;17) is specifically associated with APL and is found in virtually all cases with an M3 or an M3v FAB subtype if optimal chromosome analysis is performed *(61)*. The incidence of t(15;17) in different APL series has varied between 41% in the early days of cytogenetics and 100% in recent series *(35)*. This is because t(15;17) can only be detected when chromosomes are of good quality. Furthermore, it is important that APL cells be cultivated for 24 and 48 h before chromosome preparation, as it has been shown that direct preparation yields mostly only normal metaphases, whereas abnormal metaphases increase after culturing for 24 and 48 h *(195)*.

In 1976, Golomb et al. *(196)* described for the first time an abnormal chromosome 17 in two APL patients. Rowley et al. *(61)* in 1977 found that the rearranged chromosome arose through a reciprocal translocation between the long arms of chromosomes 15 and 17. When the translocation breakpoints were cloned, it turned out that the retinoic receptor α gene (*RAR*α) on chromosome 17 and the *PML* gene on chromosome 15 are involved *(197)*. As a result of the translocation, the truncated *RAR*α gene is moved to chromosome 15, where it is fused to PML and gives rise to a *PML-RAR*α hybrid gene that can be consistently detected by RT-PCR *(198,199)*. Experiments with transgeneic mice expressing *PML-RAR*α suggest that this fusion product is not sufficient to cause APL directly, but its expression alters myeloid development, resulting in an accumulation of myeloid precursors that may be susceptible to cooperative transforming events *(200)*.

Remarkably, APL with the *PML-RAR*α rearrangement responds—albeit temporarily—to treatment with all-*trans*-retinoic acid (ATRA). After an initial transient proliferation, the APL cells differentiate, senesce, and die. The introduction of ATRA in chemotherapy protocols has improved outcome dramatically *(201,202)*. As ATRA treatment has to start immediately in all cases with clinical suspicion or cytomorphologic evidence of AML M3 or AML M3v, t(15;17)/*PML-RAR*α has to be confirmed or excluded. In our hands the FISH technique is the quickest method, as a result can be obtained within 4 h. Chromosome banding analysis has to be performed as well to detect variant translocations or additional abnormalities. PCR also has be performed at diagnosis to allow the monitoring of MRD during and after treatment. In most patients, the *PML-RAR*α transcript cannot be detected by PCR after treatment. Several clinical trials were able to show that if PCR becomes positive again, patients will relapse *(203,204)*. Data obtained using the new real-time PCR technique, allowing quantitation of MRD, show a decrease of *PML-RAR*α copy numbers during therapy and an increase at the time of relapse *(205)*.

7.1.3. inv(16)(p13q22)/t(16;16)(p13;q22)/*CBF*β-*MYH11*

There is a close association between inv(16) and its variant t(16;16) in AML M4 with abnormal eosinophils *(62)*. Although different FAB subtypes have been reported in patients with inv(16), there is compelling evidence that AMLM4eo/inv(16) is a biologic entity. On the molecular level, the smooth muscle myosin heavy-chain gene (*MYH11*) on 16p13 is fused to the *CBF*β gene on 16q22 *(206,207)*. The fusion transcript is detectable in all patients with AML M4eo by RT-PCR *(208)*. Data on whether RT-PCR can detect more patients with t(8;21)/*AML1-ETO* or inv(16)/t(16;16)/*CBF*β-*MYH11* than chromosome banding analyses are conflicting. Results from our own group, obtained by screening 250 AML patients for *AML1-ETO* and *CBF*β-*MYH11*, showed no discrepancy between results of cytogenetics and RT-PCR (unpublished observations). These results correspond to data from the CALGB but are in contrast to results published by Langabeer et al. and Ritter et al. *(209–211)*. This discrepancy may reflect several factors, including number and quality of metaphases obtained for cytogenetic analysis.

The *CBF*β-*MYH11* fusion gene seems to have a dominant-negative function, as knocked-in mice heterozygous for *CBF*β-*MYH11* have a phenotype very similiar to that resulting from homozygous deletions of *CBF*β *(212)*. The prognostic impact of inv(16) is still in question. Some data from clinical studies show a favorable outcome, whereas in other studies only an intermediate prognosis was observed *(168,175–177,213)*.

7.1.4. 11q23 Abnormalities/*MLL* Rearrangements

The *MLL* gene is involved in more than 30 different translocations identified at the present time *(214)*. Nineteen of these translocations have already been cloned, and all lead to in-frame fusions. The partner genes do not appear to have any unifying characteristics that would clarify their role in the leukemogenic process, although a few share similiar domains. In addition to translocations, *MLL* is involved in a partial tandem duplication *(181,182)*. The function of the intact *MLL* protein in mammalian cells is only poorly understood, and how *MLL* is involved in the leukemic process remains unknown *(1)*.

The most common translocations affecting the *MLL* gene in AML are t(9;11)(p21;q23) *(215)*, t(6;11)(q27;q23) *(216)*, and t(11;19)(q23;p13) *(217)*. They are usually observed in the M5a, M5b, or M4 FAB subtypes. In therapy-related AML, 11q23 abnormalities are frequently observed, especially after treatment with one of the epipodophyllotoxins *(218)*. Patients with 11q23 abnormalities seem to have an intermediate to poor prognosis, with generally high remission rates but short survivals. Recent data from Mrózek et al. *(219)* show that significant differences in clinical outcome may exist within this cytogenetic category, in that patients with t(9;11)(p22;q23) had a significantly better outcome than cases with other translocations involving 11q23 *(219)*. Also, in children, t(9;11) seems to be associated with a better prognosis than other 11q23 rearrangements *(184)*.

7.1.5. inv(3)(q21q26)/t(3;3)(q21;q26)

Abnormalities involving breakpoints at both 3q21 and 3q26 have been described in AML in all FAB subtypes, except for AML M3/M3v and AML M4eo. Although no association with a distinct FAB subtype exists, these patients often show a typical bone marrow morphology, with an increased number of megakaryocytes and numerous micromegakaryocytes. In the

peripheral blood, normal or even elevated platelet counts are often observed. Clinical outcome in these patients is very poor *(60,220)*.

7.1.6. t(6;9)(p23;q34)/*DEK-CAN*

t(6;9) has been reported in more than 50 AML patients, mostly as the sole anomaly. It is not associated with a distinct FAB subtype, although many cases have been classified as M2 or M4 *(221)*. An increase of basophils in the bone marrow was reported in about 50% of patients. On the molecular level, the *DEK* gene on 6p23 is fused to the *CAN* gene on 9q34. The function of the fusion gene is unknown, but it seems to have a nuclear localization. t(6;9) seems to be associated with an early age of onset (mean age, 38 yr) and a poor prognosis, although few data on clinical outcome are available *(222)*.

7.1.7. t(8;16)(p11;p13)

This translocation has been reported in more than 30 cases, the majority with myelomonocytic or monoblastic leukemia, especially M5b. Erythrophagocytosis was often demonstrated. Most patients are young, sometimes infants. Usually t(8;16) is the only change. Response to chemotherapy was reported to be poor *(223,224)*. The translocation t(8;16)(p11;p13) consistently disrupts two genes: *CBP* on chromosome 16p13.3 and *MOZ* on chromosome 8p11 *(225)*.

There are several other karyotypic abnormalities that have been described as primary chromosomal aberrations in AML. These include some rare balanced translocations, such as t(1;3)(p36;q21), t(1;22)(p13;q13), t(3;21)(q26;q22), t(7;11)(p15;p15), t(11;17)(q23;q25), and t(16;21)(p11;q22), which have been identified as recurrent translocations. However, only a few patients with these alterations have yet been described, so that no reliable information on prognosis is available. On the other hand, several unbalanced abnormalities including gains and losses of whole chromosomes or deletions are thought to be primary abnormalities, but in none of these have the pathogenetic mechanisms been resolved.

7.1.8. Monosomy 5/Deletion 5q

Loss of part of the long arm of chromosome 5 or total monosomy 5 show no distinctive FAB subtype preference and are usually accompanied by secondary aberrations, most often in complex aberrant karyotypes *(35)*. The incidence of *de novo* AML is around 7% of aberrant cases compared with 17% in secondary AML *(226)*. Abnormalities of chromosome 5 are associated with a poor outcome *(175–177)*. Whether this is mainly owing to the complex aberrant karyotypes associated with these changes is unclear, because they are only rarely observed as the sole abnormality.

7.1.9. Monosomy 7/Deletion 7q

After trisomy 8, monosomy 7 is the second most frequent solitary numeric abnormality in AML *(35)*. As the sole anomaly, it is found in 3% of cytogenetically abnormal cases and is observed as one of several changes—usually in complex aberrant karyotypes—in 12%. There is no preference for a particular FAB subtype. Both monosomy 7 and deletions of the long arm seem to be particularly frequent in therapy-associated AML, especially after treatment with alkylating agents *(227,228)*. Abnormalities of chromosome 7 are associated with a poor prognosis *(175–177)*. In one study, deletion of the long arm of chromosome 7 was not an unfavorable prognostic factor *(178)*.

7.1.10. Trisomy 8

A gain of chromosome 8 is the most frequent numeric abnormality in AML, occurring as a solitary change in 5% of all cytogenetically abnormal AML cases. If cases with multiple aberrations are also considered, the frequency of +8 triples *(35)*. Trisomy 8 is a frequent additional aberration in patients with t(8;21), inv(16), t(15;17), or t(9;11) and is often observed as an abnormality in complex aberrant karyotypes. The accompanying abnormalities seem to determine the prognosis. In a study of 51 patients with trisomy 8, patients with +8 as the sole cytogenetic anomaly had an intermediate prognosis and patients with +8 in addition to favorable chromosomal aberrations maintained a good clinical outcome, whereas +8 in combination with other abnormalities determined the worst prognosis (event-free survival, 37.5% vs 55.0% vs 9.0% respectively) *(229)*.

7.1.11. Deletion 9q

Deletions of the long arm of chromosome 9 can be found as the sole abnormality without a FAB subtype preference, but they are also observed as secondary changes, in particular in addition to t(8;21) and less often in addition to t(15;17) or inv(16) *(230,231)*.

7.1.12. Trisomy 11

Like other trisomies, trisomy 11 can occur as a single abnormality but is more frequently seen together with other numeric or structural cytogenetic changes. Although it is the third most common trisomy in AML, few clinical data are available on this abnormality. Isolated trisomy 11 is predominantly associated with older age, M2 and M1 FAB subtypes, and a poor response to standard chemotherapy with an overall unfavorable prognosis *(232)*. Recently, a partial tandem duplication of the *MLL* gene was reported as a recurrent molecular defect in 37.5–91% of AML cases with trisomy 11 *(182,233)*. The mechanism by which the partial tandem duplication contributes to leukemogenesis is currently unknown.

7.1.13. Trisomy 13

About 25% of patients with trisomy 13 show no other abnormality. In a review of 29 patients, all FAB subtypes except M6 and M7 were observed in patients with trisomy 13 as the sole cytogenetic change. The median age was 60 years. Patients responded well to induction therapy, but relapse occurred quickly and survival was short *(234,235)*.

7.1.14. Trisomy 21

Trisomy 21 is present in 5% of all AML patients and in less than 1% as the sole anomaly. There seems to be no FAB type specificity *(236)*.

Other rare trisomies observed as the sole abnormality in AML involve chromosomes 4, 9, or 22 *(35,37,170)*.

7.1.15. Complex Aberrant Karyotype

A group of 10–20% of patients show so-called complex aberrant karyotypes, which are associated with a very poor prognosis *(175–178,238)*. The definition of a complex aberrant karyotype varies among different study groups. Most commonly, it is defined as at least three cytogenetic abnormalities. This definition seems to be insufficient, as patients with balanced translocations such as t(8;21) or inv(16) and additional

Table 4
Cytogenetic Classification Systems of Different Clinical Study Groups for AML

Author and reference	*Favorable*	*Intermediate*	*Unfavorable*
AMLCG, 1999 *(169)*	t(8;21), t(15;17), inv(16), t(16;16)	Normal, other abnormalities	–5/5q–, –7/7q–, inv(3), 11q23, 12p,17p, complex
CALGB, 1998 *(246)*	t(8;21),inv(16), t(16;16),del(16)	Normal	Other abnormalities
Döhner, 1998[a]	t(8;21), t(15;17)	inv(16), 11q23, all other abnormalities	–5/5q–, –7/7q–, inv(3), 12p,17p
Gale et al., 1995 *(247)*	t(8;21),inv(16), t(16;16), del(16), t(15;17)	+8, +21, t(6;9), other abnormalities	t(9;22),–5,–7, del(11)
SWOG, 1997 *(248)*	t(8;21),inv(16), t(16;16),+14	Normal, other abnormalities	–5/5q–, –7/7q–, +13, inv(3), 11q23, 17p, 20q–,+13, dm, HSR, complex
EORTC[a]	t(8;21), inv(16)		–5/5q–, –7/7q–, 11q23, complex
MRC, 1998 *(168)*	t(8;21), t(15;17), inv(16)	Normal,+8,+21,+22,del(7q), del(9q), abn(11q23), all other numeric or structural abnormalities	–5/5q–, –7, abn 3q, complex

[a]Personal communication or protocol of the study group.

aberrations fulfill this criterion as well but belong to completely different biologic entities. The "real" complex aberrant karyotype shows unbalanced karyotypic abnormalities. The incidence of complex aberrant karyotypes is age-dependent. The incidence in patients younger than 60 yr is <10%, while complex aberrant karyotypes are found in up to 20% of patients older than 60 years. Prognosis is equally poor in all age groups, with less than 10% of patients surviving longer than 1 yr *(238)*.

7.2. Secondary Chromosomal Abnormalities

Secondary chromosomal aberrations are rarely or never found alone, rather, they develop in cells already carrying a primary abnormality. Although less specific than the primary changes, secondary aberrations nevertheless demonstrate nonrandom features with distribution patterns that appear to depend on the primary abnormality and to a lesser degree on the type of leukemia (AML or ALL) *(239)*. In contrast to primary aberrations, which are often balanced rearrangements, such as translocations or inversions, common secondary aberrations almost exclusively lead to genomic imbalances (gains and losses of whole chromosomes, deletions, or unbalanced translocations).

The biologic and clinical significance of particular secondary aberrations associated with specific primary changes in AML is largely unexplored. Published data on the prognostic impact of secondary aberrations are conflicting. Although no influence on prognosis of secondary abnormalities in patients with t(8;21)(q22), inv(16)(p13q22), or t(15;17)(q22;q12) was reported in the large Medical Research Council 10 (MRC10) trial and some smaller studies *(229,240–244)*, a negative prognostic impact of additional abnormalities was noted for patients with t(8;21) and t(15;17) in one study each *(241,245)*. The European 11q23 Workshop analyzed 125 patients with t(9;11)(p22;q23). Additional chromosomal abnormalities did not impair prognosis *(215)*. More studies are needed to assess the clinical consequences of secondary aberrations in AML. Since the number of patients representing one distinct entity is usually too small for meaningful clinical comparisons, intergroup analysis affords the only available means by which to answer these important questions.

7.3. Implications of Chromosomal Abnormalities for Prognosis

The karyotype of the leukemic blast cell is the most important independent prognostic factor in AML. For clinical purposes, karyotype analysis allows one to discriminate among three major prognostic groups. A favorable outcome under currently used treatment regimens was observed in several studies in patients with t(8;21)(q22;q22), inv(16)(p13q22), or t(15;17)(q22;q11-12). Chromosomal aberrations with an unfavorable clinical course include –5/del(5q), –7/del(7q), inv(3)/t(3;3), and a complex aberrant karyotype. The remainder are assigned to an intermediate prognostic group. This group is highly heterogeneous because it includes patients with a normal karyotype and rare chromosome aberrations with a yet unknown prognostic impact. This group will need further subdivision in the future.

There is as yet no consensus concerning the final details among large clinical study groups on how to classify AML patients according to karyotype and prognosis. Different groups assign cytogenetic categories to different prognostic subgroups according to their experience (Table 4) *(168,169,246–248)*. It has to be kept in mind that treatment itself influences the impact of prognostic parameters. The major objective for the future is to find the best therapy for each biologic entity. To reach this objective, the biologic entities have to be clearly defined, and large well-designed prospective trials are needed to allow a randomized comparison of different treatment strategies even in small subgroups. For APL, this goal has already been achieved. There is worldwide agreement to treat this subgroup of patients within separate trials, implementing ATRA.

Data from Bloomfield et al. *(246)* suggest that patients with t(8;21) or inv(16) benefit from treatment with high-dose cytarabine. Compared with other cytogenetic risk groups, patients with t(8;21) or inv(16) had the best outcome overall and demonstrated the greatest benefit from increasing doses of cytarabine *(246)*. These data also stress that different treatment strategies can influence the prognosis of distinct cytogenetic subgroups. One important finding concerning cytogenetic

abnormalities and prognosis was that the incidence of distinct chromosome abnormalities varies with age, whereas the prognosis of defined cytogenetic aberrations is independent of age *(249)*.

8. FAB CLASSIFICATION OF ALL

The lineage assignment, subclassification of precursor B- or T-cell leukemia, and stratification of treatment according to cell-biologic risk groups in ALL are in the domain of immunophenotyping and cyto-/molecular genetic analyses. The morphologic categories L1–L3, originally proposed by the FAB group, no longer have clinical importance, with the exception of the L3 subtype *(3,14,250)*. Even in cases that display the L3 morphology (relatively uniform blasts with intensively basophilic cytoplasm and sharply defined, fat-containing vacuoles) the results of cytogenetic analysis and immunophenotyping should be considered before a definite diagnosis of L3-type Burkitt cell leukemia is made. The morphologic appearance of L3 can be imitated by the AML subtypes M0, M1, or M5 or even by several undifferentiated solid tumors. Also, in rare cases of L1- and L2-ALL, vacuolation can be seen *(14)*.

In some cases of ALL, >40% of the lymphoblasts have a hand-mirror shape, but this feature has also been described in rare instances of AML. At present, this morphologic finding merely seems to identify morphologic variants without distinguishing clinical correlations *(14)*.

It has been suggested that a number of *BCR-ABL*-positive ALL cases show a unique morphologic appearance: in addition to the dominant lymphoid blast cell population, there are larger blasts with myeloid characteristics, some of the latter even showing a positive MPO reaction (mostly <3%). This may be confused in some cases with the diagnosis of AML M0 or even AML M1 with the Philadelphia translocation *(14)*. Conversely, some AML are Philadelphia chromosome-positive *(251)*.

9. IMMUNOPHENOTYPING OF ALL

Since the demonstration by Borella and Sen *(252)* that in some children with ALL, leukemic lymphoblasts are of thymic origin, immunophenotyping has become essential in the diagnosis of ALL and has substantially contributed to a more precise and biologically oriented classification of the disease (reviewed in refs. *8*, *22*, *23*, *26*, and *253–255*). During the last two decades, immunophenotyping in ALL, initially performed with polyclonal antisera and subsequently with a rapidly expanding panel of MAbs, has mainly been applied to distinguishing ALL from AML, lineage assignment of leukemic blasts, phenotypic characterization of pathologic cell subsets, and examining the role of membrane antigen expression in predicting treatment response (reviewed in refs. *8*, *9*, *23*, *25*, *254*). Additionally, based on observations that leukemic blasts frequently show aberrant or asynchronous antigen expression compared with normal hematopoietic cell differentiation, leukemia-associated phenotypic features have been routinely used to detect MRD in ALL (reviewed in ref. *27*). More recently, immunophenotyping in conjunction with cytogenetic and molecular genetic studies has identified biologically and clinically distinct subsets within the major diagnostic subgroups of precursor B- and T-cell ALL and has become decisive in monitoring risk groups in therapeutic studies (reviewed in refs. *8* and *254–256*).

Current procedures for the diagnosis, lineage affiliation, and characterization of maturational stages of ALL are outlined in Fig. 1. It should be emphasized that both the lineage affiliation and the definition of maturational stage in ALL are based on patterns of antigen expression demonstrated by an appropriate selection of CD MAbs rather than on the presence or absence of a single antigen. In addition, it is noteworthy that the dominant phenotype of a leukemic cell population reflects the degree of maturation achieved by a leukemic clone and may not correspond to the initial target cell of the disease, mostly a more immature progenitor cell.

The following sections briefly discuss significant associations between immunophenotypic features and numeric and/or structural chromosomal abnormalities that have recently contributed to a refined ALL classification, especially in precursor B-cell ALL. It should be noted that accurate phenotypic predictions of specific translocations in precursor B-cell ALL could not be obtained by simply classifying antigen expression as either positive or negative but that they require more complex descriptions of patterns of expression or combinations of antigens.

9.1. B-Cell Precursor ALL

9.1.1. t(4;11)(q21;q23)

The t(4;11)(q21;q23) chromosomal abnormality occurs in about 2–6% of both children and adults with ALL and has been associated with characteristic immunophenotypic and clinical features [e.g., high leukocyte counts, predominance of females in infants, frequent organ enlargement, and increased incidence of central nervous system (CNS) leukemia at diagnosis); reviewed in refs. *257* and *258*]. Previous reports, mainly in infant ALL, have suggested that t(4;11)-associated acute leukemias mostly originate in multipotent or very early CD10-negative B-progenitor cells with a high frequency of myeloid-antigen positivity *(259,260)*. Recent studies have analyzed the immunophenotypic and genotypic features of this subgroup in greater detail. In the vast majority of ALL cases with t(4;11), leukemic blasts show a typical antigenic profile (e.g., CD19+, CD10–, CD24– or weakly +, cyIgM – or +, CD15 and/or CD65s+) indicative of an immature pro-B phenotype with frequent coexpression of particular myeloid antigens (i.e., CD15, CD65s). This clear-cut association of immunophenotypic features with t(4;11), initially described in infant ALL *(261–263)*, has also been recently found in adult patients *(264–270)*. Southern blot analysis revealed Ig heavy-chain gene rearrangements in virtually all cases as well as oligoclonal disease in some of them *(261)*, thus underlining the early B-cell commitment of blast cells with this cytogenetic abnormality. Based on our experience in a large series of childhood and adult ALL patients with 11q23 rearrangements *(261,268,271,272)*, these features, especially the missing or weak expression of CD24 compared with CD19, and the coexpression of CD65s, usually associated with negativity of other panmyeloid antigens (e.g., CD13, CD33), are highly predictive for the cytogenetic and/or molecular demonstration of *MLL* rearrangements, mostly owing to a t(4;11), or more rarely, other 11q23 aberrations. More recently, the 7.1 MAb, which recognizes a specific an-

tigen of the chondroitin sulfate proteoglycan family, has been demonstrated to detect with high sensitivity childhood ALL with *MLL* rearrangements, but it does not distinguish between the different translocation partners involved in *MLL* rearrangements *(133,273)*, thus not obviating the need for molecular genetic analyses. Our own results in a large number of childhood and adult ALL patients suggest that carefully constructed antibody panels, including 7.1, may be helpful for the identification of ALL carrying *MLL* rearrangements and for detection of MRD by flow cytometry *(133)*.

9.1.2. Philadelphia Translocation

The Philadelphia (Ph) translocation, or t(9;22)(q34;q11), occurring in 15–30% of adults and 3–5% of children with ALL, is usually associated with a common or pre-B ALL phenotype *(264,274–283)*. Some studies have identified a low proportion of immature CD10-precursor B-cell, B- or pure T-lineage features in Ph+ ALL *(274,284)*. Recent data have indicated that, similar to chronic myelogenous leukemia, a primitive hematopoietic cell is the target for the leukemic transformation in Ph+ ALL *(285)*. Although several immunophenotypic features, such as increased myeloid antigen expression *(267,282)*, positivity of the KOR-SA3544 antigen, *(286)*, coexpression of CD34 and CD10 *(270)*, and expression of CD25 *(280)* have been suggested to be associated with Ph+ ALL, there does not appear to be a definitive correlation between this translocation and immunophenotype. Our data in childhood and adult ALL *(279,287)*, as well as the findings of others *(288,289)*, suggest that myeloid antigens are not coexpressed more frequently in Ph+ ALL than in Ph– ALL cases. No extensive analyses have yet been performed to investigate the correlation between surface antigen expression and the two different breakpoint cluster regions (major or minor bcr) detected on chromosome 22 in Ph+ ALL. Preliminary results in adult ALL suggested that rearrangements in the major bcr are more common in My+ than in My– ALL *(279)*.

The monoclonal antibody KOR-SA3544 was previously reported to recognize Ph+ ALL with high sensitivity *(286)*. More recent studies, however, have shown that this antibody specifically recognizes the nonspecific crossreacting antigen (NCA)-50/90 (CD66c), one of the carcinoembryonic antigen (CEA)-related glycoproteins *(290)*, and reacts with distinct subsets of precursor B-cell ALL, including Ph+ ALL, *ETV6-AML1*-negative ALL, and hyperdiploid ALL *(291,292)*.

9.1.3. *ETV6-AML1*

The *ETV6-AML1* fusion gene, created by t(12;21), is the most common translocation in childhood ALL, occurring in about 20–25% of cases, and is present in <3% of adult ALL cases. The *ETV6-AML1* rearrangement is restricted to patients with nonhyperdiploid precursor B-cell ALL, and most cases display a common ALL or, less frequently, a pre-B ALL phenotype. The high frequency of myeloid antigen expression (i.e., CD13 and/or CD33) originally reported in a large series of children with *ETV6-AML1* rearrangement enrolled in the German and Italian multicenter trials *(293)* was confirmed by other reports *(294–296)*. Interestingly, other characteristic immunophenotypic features have recently been described that are highly predictive of *ETV6-AML1* rearrangement and may be used as a screening test for this genetic abnormality *(296)*. These include negativity of CD66c *(292)* and complete or partial lack of both CD9 and CD20 expression *(296,297)*.

9.1.4. t(1;19)(q23;p13)

Previous studies have indicated that t(1;19)(q23;p13), found in 5–6% of childhood and <5% of adult ALL cases, is strongly associated with cytoplasmic μ+ pre-B ALL *(298)*. Although subsequent studies confirmed the close association between t(1;19) and pre-B phenotype in both childhood and adult ALL *(299)*, this abnormality was also detected in some cytoplasmic μ– common ALL *(274,299,300)*. More recently, some authors described a pattern of surface antigen expression (i.e., CD9+, CD19+, CD22+, CD20±, CD34–, $CD45^{high}$) that is characteristic of ALL with t(1;19) but lacks specificity *(301,302)*. Polyclonal and monoclonal antibodies are now available for the flow-cytometric detection of the E2A-PBX1 protein in the nucleus of t(1;19)+ leukemic blasts *(303)*.

9.2. B-ALL

While in precursor B- and T-cell ALL, specific cytogenetic abnormalities do not show a close relationship to the FAB subtype, leukemic blasts in mature surface immunoglobulin (sIg)+ B-ALL are usually characterized by FAB L3 morphology and invariably exhibit t(8;14)(q24;q32) (in 75–85% of the patients) or one of the variant translocations t(2;8)(p11-12;q24) and t(8;22)(q24;q11). The high predictive value of these B-ALL-associated chromosomal anomalies for L3 morphology has recently been demonstrated *(37)*. It should be noted, however, that exceptions to these associations have been described. These include pediatric ALL patients with the 8;14 translocation, FAB L3 morphology, and clinical and laboratory features consistent with B-cell ALL, whose leukemic blasts displayed a less differentiated B-precursor immunophenotype *(304)* or lacked surface and cytoplasmic Ig *(305)*, and adults with sIg light-chain-positive ALL of L1 or L2 morphology without t(8;14) or its variants *(306)*. These unusual findings demonstrate the importance of evaluating patients with a combination of diagnostic tools in order to identify those who do not fit the recognized subgroups for a given disease. Furthermore, clinical behavior and outcome data in these patients suggest a hierarchy of clinical relevance of laboratory tests, with cytogenetic evidence of t(8;14) or one of the variant translocations being of greatest importance for assigning patients to B-cell ALL-specific protocols *(304)*; treatment outcome of other subtypes without one of these three translocations appears to be similar to those for precursor B-cell ALL.

9.3. T-Lineage ALL

Similar to precursor B-cell ALL, precursor T-cell ALL is manifested by a strong interpatient biologic heterogeneity, and leukemic transformation may occur at distinct stages of T-cell ontogeny, which results in lymphoblasts with immunophenotypic features corresponding to immature or more mature T-cell progenitors. In marked contrast to precursor B- or mature B-cell ALL, however, clear-cut relationships have not yet been established between specific chromosomal changes, occurring in about 44–61% of pediatric T-lineage ALL patients *(307,308)* and the maturational stage of T-ALL blasts or a particular pattern of surface antigen expression *(281,307–310)*.

Moreover, it has not been possible to identify characteristic phenotypic features within groups of frequent chromosomal abnormalities in T-ALL *(307,309)*, except for children with t(11;14), whose leukemic blasts expressed a profile of membrane surface antigens (i.e., CD4+, CD8+, CD3±) that has been associated with more mature thymocytes *(309,311)*. Most of these data were derived from cytogenetic analyses in childhood ALL, and detailed results are not yet available as to the correlation of immunophenotype and karyotype in adult patients with precursor T-cell ALL.

Alterations of the *TAL1* protooncogene on chromosome 1p32, either by translocation or other rearrangements, have recently been shown to represent the most common nonrandom genetic defect associated with precursor T-cell ALL, occurring in about 10–25% of patients *(312–314)*. Several reports unanimously demonstrated that *TAL1* alteration in T-ALL exclusively occurred in CD3– or CD3+ T-ALL of the αβ lineage, whereas no clear association of *TAL1* gene rearrangements with a distinct stage of thymocyte maturation could be detected *(312–315)*.

10. PROGNOSTIC IMPACT OF IMMUNOPHENOTYPING IN ALL

The lack of standardized criteria in the past for the classification of immunophenotypic subgroups, the paucity of controlled prospective studies on the treatment outcome of precursor B- and T-cell ALL subsets, and the different treatment strategies administered complicate the assessment of the prognostic impact of immunophenotyping studies in ALL. In addition, the strong correlation between certain immunophenotypic subgroups and cytogenetic or clinical features (see above) has called into question the value of immunophenotyping as an independent predictor of treatment outcome. Finally, several studies have shown that the prognostic impact of immunophenotypic subgroups as well as chromosomal abnormalities is diminished by the improved efficacy of chemotherapy; hence, prognostic factors must be evaluated in the context of the therapy delivered *(270,299,316–319)*.

In precursor B-cell ALL, no substantial differences in remission rates were recorded for immunophenotypic subgroups, but several studies revealed an association between the maturational stage of B-lymphoblasts and the duration of remission. Most studies in both childhood and adult ALL have reported a worse prognosis for patients whose leukemic blasts express an immature CD10-negative pro-B phenotype, also referred to as early pre-B or null-ALL *(254,269,270,320–323)*, which, however, was frequently associated with adverse biologic (e.g., 11q23 rearrangements) and clinical features (e.g., high tumor burden, age < 1 yr).

Cytogenetic and molecular genetic studies have provided conclusive evidence that children and adults with common and pre-B ALL differ significantly with respect to the incidence of the known favorable or unfavorable chromosomal translocations. For instance, t(9;22) accounts for up to 55% of adult and <5% of children with CD10+ precursor B-cell ALL, whereas the reported frequency for t(12;21), associated with a good prognosis in most recent studies, ranges between 12 and 36% in childhood common or pre-B ALL and rarely occurs in adult patients *(264,275,289)* (reviewed in ref. *324*). These findings may partially explain the striking differences observed in treatment outcome between children and adults with common or pre-B ALL.

Confirmation of the prognostic importance of the pre-B ALL immunophenotype has been limited to sequential studies of the Pediatric Oncology Group (POG), since, until recently, this was the only group performing cytoplasmic μ testing in the context of large prospective clinical trials. Previous studies of the POG suggested that the pre-B phenotype might be an independent prognostic marker for reduced event-free survival *(298)*. However, more recent data show that only the subgroup of children with pre-B ALL and t(1;19) has a worse treatment outcome *(325)*. By contrast, the German ALL-BFM trials, the analysis of the MRC UKALL Trial XI, and a single-center study did not reveal any significant differences in remission duration between common and pre-B ALL *(320,326)*.

In children with precursor B-cell ALL, prognosis has been linked to other immunophenotypic features, such as CD20, CD34, and CD45 expression, and it has been suggested that the lack of CD20 and CD45 antigens or the presence of CD34 on leukemic blasts may be associated with a longer event-free survival *(327–330)*. However, in view of the relationship of these immunophenotypic features (e.g., absent CD45) to other biologically favorable characteristics *(297,329,331)*, their prognostic significance has to be evaluated in further studies by adjusting results for the presence of other risk factors.

Several studies in childhood and adult ALL have shown that a remarkable prognostic improvement of B-ALL is achieved by the development of intensive treatment strategies, especially adapted to the biologic and clinical features of this disease *(318,319)*. These data impressively illustrate that more effective treatment can offset the negative prognostic impact of biologic characteristics, such as the immunophenotype or chromosomal translocations.

In T-lineage ALL, various immunophenotypic features seem to be associated with an increased risk of treatment failure, including an immature pro-/pre-T-ALL phenotype, membrane expression of CD3 or MHC class II antigen, and negativity of CD2, CD5, THY antigen (similar to CD1), or CD10 *(254, 320,327,332–336)*. The prognostic impact of these factors, however, has differed according to the treatment strategies used, and immunophenotyping still represents a controversial prognostic factor that has not been routinely used for risk classification or assignment to novel treatment strategies in high-risk precursor T-cell ALL patients.

In our experience and in other studies, children and adults with pre-T ALL differ markedly with respect to their phenotypic and genotypic features, suggesting an arrest of adult pro-/pre-T ALL at a less mature differentiation stage than in childhood, which may be closely related to the worse treatment outcome observed in these patients *(327,332,334,337,338)*. Similar results have previously been published in adolescent and adult patients, whose leukemic blasts showed CD7 antigen expression with an absence of myeloid, B-, or more mature T-cell differentiation antigens *(339)*. Most interestingly, these leukemic blasts were capable of multilineage differentiation in vitro both spontaneously and after stimulation with appropriate cytokines, sug-

Table 5
Frequency of Ploidy Groups: Adult versus Childhood ALL

	Frequency (%)	
Ploidy group	*Adult ALL*	*Childhood ALL*
Normal karyotype	26–34	8–56
Hypodiploidy < 46	2–8	5–7
Pseudodiploidy	7–59	3–42
Hyperdiploidy 47–50	7–17	8–16
Hyperdiploidy > 50	4–9	14–28
Near-triploidy	3	<1
Near-tetraploidy	2	1

Table 6
Chromosomal Abnormalities in ALL

			Frequency	
Abnormality	*Disease subtype*	*Fusion gene*	*Childhood (%)*	*Adult (%)*
t(1;19)(q23;p13)	Pre-B-ALL	*E2A-PBX1*	5–6	3
t(4;11)(q21;q23)	Pro-B ALL	*MLL-AF4*	2	6
t(9;22)(q34;q11)	c-ALL	*BCR-ABL*	2–5	25–30
t(8;14)(q24;q32)	B-ALL	*MYC-IGH*	3	5
t(10;14)(q24;q11)	T-ALL	*HOX11-TCR*	1	3
t(12;21)(p13;q22)	Pre-B-ALL	*ETV6-AML1*	10–20	<1
9p	T-, pre-B-ALL	$p16^{INK4A}$	7–12	15
6q	c-, pre-B-,T-ALL	?	4–13	6
14q11	T-ALL	*TCR*	1	6

gesting that acute leukemia in these patients evolved from in vivo transformation of immature pluripotent hematopoietic cells that respond poorly to conventional chemotherapy. Consistent with this hypothesis, patients with immature precursor T-cell ALL showed more frequent coexpression of CD34, CD117, and/or myeloid antigens *(327,338,340)*.

Further attempts to identify additional prognostically relevant subgroups of precursor T-cell ALL have been largely unsuccessful in both childhood and adult ALL *(332,341)*. However, at least three multicenter trials in childhood ALL, using similar maturational staging systems, have recently lent strong support to evidence that children with cortical (CD1a+) precursor T-cell ALL have a better early response to treatment, as illustrated, for instance, by the in vivo response to corticosteroids, and a significantly longer duration of event-free survival than in those with an immature or mature precursor T-cell phenotype *(310,320,342)*. Similar data, showing a significant improvement in survival of adult patients with CD1, CD2, CD4, and CD5 expression compared with patients not expressing these antigens, have been recently published by the Cancer and Leukemia Group *(270)*. Although, it is unclear at present why patients with a cortical immunophenotype respond better to treatment, recent investigations of apoptosis-related parameters, including spontaneous apoptosis in vitro and modulation of apoptosis by interleukin-7, suggested that maturational stages of precursor T-cell ALL may differ as to their accessibility to apoptotic programs, with lymphoblasts expressing CD1a or exhibiting a selection-related phenotype being more susceptible to apoptosis than leukemic lymphoblasts with an immature phenotype *(342,343)*.

Several studies have suggested that a subclassification of membrane CD3+ precursor T-cell ALL according to T-cell antigen receptor (TCR) αβ or γδ expression provides valuable clinical information, because TCR-γδ+ cases represent an important, albeit rare, subgroup of precursor T-cell ALL with distinctive clinicopathologic features and prognosis *(344–346)*. Further prospective studies are needed to characterize more thoroughly the cell-biologic features of TCR-γδ+ lymphoblasts and to confirm the better prognosis of this subgroup compared with TCR-αβ+ precursor T-cell ALL *(346)*.

11. GENETIC ABNORMALITIES IN ALL

Clonal karyotypic abnormalities are found in 60–85% of ALL cases *(172,267,281,347)*. The Third International Workshop on Chromosomes in Leukemia found the majority of cytogenetic changes in cases with B-cell precursor ALL, with only 39% occurring in T-cell ALL. In children, an abnormal karyotype was observed in 61% of T-ALL cases *(308)*.

Gross numeric disturbances, in particular, massive hyperdiploidy, are more frequent in ALL than in AML. More than 30% of cases are hyperdiploid and around 10% hypodiploid. More than 30 different recurring chromosome aberrations are known in ALL. Taken together, these aberrations are present in two-thirds of all cytogenetically abnormal ALL cases. Some of them are of particular clinical importance or are specifically associated with distinctive immunophenotypes.

The distribution of chromosomal abnormalities differs between childhood and adult ALL *(348)*. The two most striking examples are the Philadelphia translocation t(9;22)(q34;q11), which occurs in about 3–6% of cases in childhood but in 15–30% of adult cases. On the other hand, the translocation t(12;21)(p13;q22) occurs in about 10–15% of childhood ALL but is found in <3% of adults with this disease. These differences in ALL genetic features between age groups contribute to the differences in response to therapy *(349)*. However, the prognostic impact of distinct karyotypic abnormalities seems to be comparable between age groups: for example, the Philadelphia translocation is associated with the poorest outcome in childhood as well as in adult ALL. However, recent data suggest that within the subgroup with Ph+ childhood ALL, some patients, who can be identified by young age and leukocyte counts of <50.000/μL, benefit from intensive chemotherapy *(350)*.

11.1. Chromosomal Abnormalities and Their Molecular Correlates

In ALL, it is common to classify karyotypes into ploidy groups, on the one hand, and groups with specific rearrangements, on the other hand. Commonly recognized ploidy groups are low hyperdiploidy (modal number 47–50), high hyperdiploidy (>50), near-triploidy, near-tetraploidy, near-haploidy, hypoploidy (45 and lower), pseudodiploidy (normal number of chromosomes with structural changes) and normal karyotype (Table 5) *(351)*. An array of nonrandom structural rearrangements has been described (Table 6).

11.1.1. Hypoploidy <46 Chromosomes

Modal chromosome numbers of 45 and less are rare, especially the near-haploid numbers of 24–36. Commonly lost are chromosomes 1, 5, 6, 10, 11, 18, 19, 21, and 22. Near-haploid

cases almost always have only numeric changes. Many cases also have a hyperdiploid population twice the near-haploid number. Hypoploidy confers a rather poor prognosis in adults as well as children with ALL *(281,352)*.

11.1.2. Pseudodiploidy

A normal chromosome number with structural changes is the most frequently found abnormal karyotype in ALL. Nearly two-thirds of cases have recurrent translocations. Most cases have structural changes with only a few combining structural and numeric aberrations. The prognosis depends on the specific chromosome rearrangements rather than on chromosome number in this subgroup. More accurate identification of underlying specific structural aberrations will make the pseudodiploid group superfluous in a prognostically useful classification.

11.1.3. Hyperdiploidy 47–50 Chromosomes

Somewhat less than one-fifth of all ALL cases belong to this category. Most cases have 47 or 48 chromosomes. About one-fourth of cases show only numeric changes, whereas in the others, the pattern of structural abnormalities is similar to that of ALL in general. The chromosomes mainly gained in this group are X, 5, 8, 10, and 21 *(347)*. In childhood, numbers between 47 and 50 are associated with an intermediate prognosis.

11.1.4. Hyperdiploidy >50 Chromosomes

Chromosome numbers cluster around 51–55. There seems to be a certain pattern of gained chromosomes. Frequently gained chromosomes are 4, 6, 8, 10, 14, 17, and 21. About half of all childhood and adult ALL cases with hyperdiploidy >50 show additional structural chromosomal rearrangements, with t(9;22)(q34;q11) being the most common in adults *(353)*. In children with ALL, a modal number between 51 and 55 is associated with a favorable prognosis. The combination of trisomies of both chromosomes 4 and 10 with hyperdiploid ALL identifies a subgroup of patients with an extremely favorable outcome and a strong likelihood of cure with antimetabolite-based chemotherapy *(353)*. In adult ALL, the impact of hyperdiploidy on prognosis appears to be less significant. This is mainly because hyperdiploidy in adulthood is often accompanied by a t(9;22)(q34;q11) *(267,354)*. Poor-risk structural rearrangements override the prognostic impact of otherwise favorable numeric changes. If adults who show only numeric changes and a modal number >50 are taken into account, the prognosis is favorable.

11.1.5. Near-Triploidy and Near-Tetraploidy

Near-triploidy and near-tetraploidy are more frequent in adult ALL than in childhood ALL. Although near-triploidy seems to be associated with a poor outcome, the prognosis associated with near-tetraploidy is in the range of that conferred by a normal karyotype. These data have to be interpreted cautiously because the number of reported cases is still small.

11.1.6. Single Chromosome Gains and Losses

The incidence of a trisomy or monosomy as the only karyotypic anomaly in ALL is low. In childhood ALL, the most common changes are trisomy 8, monosomy 20, and trisomy 21. In adults, the data are scanty, so that the significance of monosomies or trisomies as isolated karyotypic changes is unclear.

11.1.7. t(12;21)(p13;q22)/*ETV6-AML1*

The translocation t(12;21) was long considered a rarity, since it was detected in <0.05% of patients analyzed by chromosome banding *(347)*. However, this rearrangement is difficult to detect, because only small segments are translocated. After the *ETV6* (or *TEL*), and *AML1* (also rearranged in AML) genes were cloned, molecular tools for the detection of *ETV6-AML1* rearrangement became available *(355)*. With molecular techniques such as FISH, Southern blot, and RT-PCR, *ETV6-AML1* fusion transcripts were detected in up to 27% of children, making it the most common genetic abnormality in pediatric ALL *(356,357)*. Interestingly, the nonrearranged *ETV6* allele seems to be deleted in almost all t(12;21) cases. The *ETV6-AML1* rearrangement is associated with a B-cell precursor immunophenotype and confers an excellent prognosis *(293)*. In adult ALL, the frequency of *ETV6-AML1* rearrangements is low (3–4%) and little is known about its prognostic significance *(358,359)*.

11.1.8. t(9;22)(q34;q11)/*BCR-ABL*

The Philadelphia translocation is the most frequent rearrangement in adult ALL. Its incidence is age-dependent. In childhood ALL, it occurs in about 3–6% of cases, whereas in adults, the frequency is at least 15–30% *(267,360)*. Owing to the reciprocal translocation between the long arms of chromosome 9 and 22, the large 3' segment of the *ABL* gene from chromosome 9 is translocated to the 5' part of the *BCR* gene on chromosome 22, creating a hybrid *BCR-ABL* gene that is transcribed into a chimeric *BCR-ABL* mRNA, which generates a protein with an increased tyrosine phosphokinase activity compared with normal human c-*ABL*. Depending on the breakpoint within the *BCR* gene, two different fusion proteins can occur. If the break takes place in the major breakpoint cluster region (M-bcr), a fusion protein of 210 kDa, called $p210^{BCR\text{-}ABL}$, is created. This fusion protein is observed in about 50% of adult Ph+ ALL and 20% of childhood Ph+ ALL cases. The remaining patients show a break within the minor breakpoint cluster region (m-bcr), translating into a smaller *BCR-ABL* fusion protein called $p190^{BCR\text{-}ABL}$ *(361)*. In vitro studies show that $p190^{BCR\text{-}ABL}$ is a more active tyrosine kinase than $p210^{BCR\text{-}ABL}$. An association between breakpoint location and clinical features and prognosis has not been found so far *(264,362)*. Only one study analyzing 36 patients with Ph+ ALL after bone marrow transplantation reports that the expression of $p190^{BCR\text{-}ABL}$ was associated with a higher risk of relapse than was the expression of $p210^{BCR\text{-}ABL}$ *(363)*. Some rare breakpoints also exist within the *BCR* gene, which lead to proteins of different sizes.

Chromosome banding analysis seems to underestimate the incidence of the *BCR-ABL* fusion gene, and Ph-negative cytogenetics with positive tests for the *BCR-ABL* fusion gene have been documented, although in rare instances *(364–366)*. Molecular tools for the detection of the *BCR-ABL* rearrangement include FISH and PCR, which are complementary to cytogenetics. The necessity for molecular screening for the *BCR-ABL* rearrangement in B-lineage ALL is debatable and largely depends on the availability and quality of chromosome banding analysis. Considering the high prognostic impact of the *BCR-ABL* rearrangement, screening with either FISH or PCR is justified, especially if only suboptimal chromosome banding analysis is available or a normal karyotype was observed.

The prognosis of Ph+ ALL is poor in childhood *(367)* as well as in adults *(267,281,360)*. Recent studies show that in childhood ALL a subgroup of patients seems to benefit from intensive chemotherapy *(283,350)*. A study in adult ALL was able to show that karyotypic abnormalities in addition to the Ph translocation have prognostic impact *(279)*.

11.1.9. 11q23/*MLL* Abnormalities

A common molecular event is the disruption of the *MLL* gene (mixed-lineage leukemia or myeloid-lymphoid leukemia) located on chromosome 11, band 11q23. *MLL* rearrangements occur in ALL as well as in AML. More than 30 chromosomal loci are known to participate in *MLL* rearrangements *(214)*. Although some patterns and associations between the partner genes are beginning to emerge, it is not yet possible to frame a single hypothesis for 11q23 leukemic transformation. In ALL, the most commonly involved partner genes of *MLL* are *AF4* (4q21) and *ENL* (19p13).

The translocation t(4;11)(q21;q23) was first described by Oshimura et al. in 1977. It is observed in >60% of infants with ALL, 2% of children, and 3–6% of adults *(262,281)*. It is associated with young age (generally under 2 yr), female sex, and high white blood cell counts, and is frequently associated with organomegaly and CNS involvement.

The clinical outcome for both adults and children with t(4;11)(q21;q23) has been poor regardless of age *(262, 267,281,368)*. However, intensifying treatment strategies seems to improve prognosis substantially. Although data in adult ALL with t(4;11) are still scarce, risk-adapted therapy might benefit this group, as it did in children *(268)*.

11.1.10. t(1;19)(q23;p13)/*E2A-PBX1*

This translocation was first described in 1984 by Carroll et al *(369)*. Subsequent studies confirmed it as one of the most common translocations in childhood ALL, with an incidence of 5–6% overall and 25% of pre-B cell-ALL cases *(299)*. In adult ALL, it is present in <5% of cases *(267,370,371)*. Clinical characteristics include a high white blood cell count, high lactate dehydrogenase levels, and a pseudodiploid karyotype. At the molecular level, the *E2A* gene on chromosome 19 is fused to *PBX1* on chromosome 1. *E2A* encodes two transcription factors, E12 and E47, via alternative splicing, which are considered essential for normal lymphopoiesis and regulation of B-cell development. A study in pediatric ALL compared the value of chromosome banding analysis and the PCR technique for detection of the t(1;19) and found that more cases were detected by PCR. In half of these additional cases, no metaphases were obtained, whereas in the others, only normal metaphases were observed *(372)*. Improving culturing techniques can reduce these discrepancies. Pediatric studies have shown that children with t(1;19) fare poorly on standard treatment protocols but that a favorable outcome can be achieved with intensified treatment *(325,373)*. As incidence of this rearrangement is low in adults, no data on its prognostic impact are available.

11.1.11. t(8;14)(q24;q32) and Variants t(2;8)(p12;q24), and t(8;22)(q24;q11)

t(8;14) and its variants are associated with mature B-cell neoplasia with remarkable specificity. They are found in both leukemia and lymphoma of the Burkitt type. The crucial event in all three translocations is the juxtaposition of the oncogene *MYC*, located on 8q24, with either the immunglobulin heavy-chain locus (14q32), the immunglobulin light-chain locus κ (2p12), or the immunglobulin light-chain locus l (22q11). Under the influence of transcription-stimulating sequences in the constitutively active immunglobulin locus, the transcription of *MYC* is increased, ultimately leading to neoplastic growth. t(8;14) is the most common (85%) of these rearrangements, whereas t(2;8) and t(8;22) are found in around 5 and 10% of patients, respectively *(35)*.

Mature B-cell ALL was long believed to be associated with poor prognosis. The introduction of short-term dose-intensive regimens such as hyperfractionated cyclophosphamide, high-dose methotrexate, and cytarabine has significantly improved clinical outcomes in children as well as adults *(318,374, 375)*.

11.1.12. 6q Deletions

Deletions of the long arm of chromosome 6 are common in ALL, occurring in 5–10% of cases with clonal abnormalities, being the only change in about two-thirds *(35)*. PCR-based techniques to detect the loss of heterozygosity have yielded evidence that deletions of 6q seem to be more frequent when identified by this method compared with chromosome banding techniques. With molecular techniques loss of heterozygosity on the long arm of chromosome 6 occurred in up to 32% of cases *(376)*. Such deletions are not specific for ALL but rather seem to be characteristic of lymphoid malignancies. Molecular techniques have defined a minimal deleted region, but so far the putative tumor suppressor gene on the long arm of chromosome 6 has not been cloned *(376)*. Clinical studies have either confirmed no difference in prognosis or reported improved outcomes for 6q deletions *(267,354)*.

11.1.13. 9p Abnormalities

Loss of 9p material can occur owing to deletions or unbalanced translocations. The commonly deleted region was found to be 9p21-p22. From 7 to 12% of childhood and 12 to 15% of adult ALL cases show 9p abnormalities *(267,347,360)*. They occur in B-lineage as well as in T-lineage ALL. An association with high-risk clinical features such as older age, high leukocyte counts, bulky disease, and an increased incidence of CNS disease was reported *(281)*. Candidates for the putative tumor suppressor gene at 9p21 are $p16^{INK4A}$ and $p15^{INK4B}$. They both encode proteins that inhibit the cyclin-dependent kinases CDK4 and CDK6 and play a crucial role in cell cycle progression. The main mechanism for $p16^{INK4A}$ inactivation is biallelic deletions with $p15^{INK4B}$ gene codeletion in most but not all cases. Point mutations within $p16^{INK4A}$ are rare *(281)*.

11.1.14. t/dic(9;12)(p11-12;p11-13)

This rare group of chromosomal abnormalities leads to loss of parts of the short arms of chromosomes 9 and 12. The most common rearrangement in this group is dic(9;12), which is associated with young age, no CNS involvement, B-progenitor phenotype, and an excellent prognosis *(377)*.

11.1.15. 12p Deletions

Partial monosomy 12p owing to deletion or unbalanced translocations is observed in about 5% of ALL cases *(267)*. It usually occurs within complex aberrant karyotypes and only

rarely as the sole abnormality. The presence of 12p deletions seems to have no impact on prognosis *(267)*.

11.1.16. Abnormalities Involving the *T-Cell Receptor* Genes

Translocations involving the *TCR* genes are among the most common aberrations in T-cell ALL *(281)*. Most patients with T-cell ALL show a normal karyotype by cytogenetics, but those who show an abnormality have about 50% of breakpoints that map to loci of one of the *TCR* genes. These include chromosome 14 band q11 (*TCR*-α and *TCR*-δ), 7q32-36 (*TCR*-β), and 7p15 (*TCR*-γ). A number of distinct chromosomal translocations have been identified. A common theme is the juxtaposition of *TCR* promotor/enhancer elements to a variety of putative or proven transcription factors located at breakpoints on the partner chromosomes. The most common translocation in T-cell ALL is t(10;14)(q24;q11), occurring in 7–14% of ALL of T-lineage origin. The *HOX11* gene, a homeobox gene, located on 10q24, becomes transcriptionally activated. The prognosis of t(10;14) seems to be favorable in adults *(267,360,378)*. Other recurrent translocations in T-cell ALL involving TCR genes are t(8;14)(q24;q11) *(379)*, t(11;14)(p15;q11) *(311)*, t(11;14)(p13;q11) *(311)*, and t(7;9)(q34;q34) *(308,380)*.

11.1.17. *TAL1* Gene Rearrangements

Approximately 20–30% of children and 10–30% of adults with T-cell ALL have rearrangements within a gene called *TAL1* (or *TCL5* or *SCL*) on chromosome 1, band p33. Two mechanisms of rearrangements exist. About 3% occur by a balanced translocation t(1;14)(p33;q11) that results in a rearrangement between the *TAL1* gene and the *TCR*α/δ locus *(381)*. In 25–30% of pediatric patients with *TAL1* alterations, the changes are not detectable by chromosome banding analysis *(35)*. In these patients, submicroscopic deletions within chromosome band 1p33 fuse the coding exons of *TAL* to the first noncoding exon of *SIL* (SCL-interrupting locus) *(314)*. This rearrangement can be detected either by Southern blot or PCR analysis. Both mechanisms lead to a transcriptional activation of *TAL1*. Outcome seems not to differ significantly between patients with or without *TAL1* rearrangement *(281)*.

Despite the proven diagnostic and prognostic value of cytogenetic analyses in ALL, there are compelling reasons to add molecular techniques. In some cases, clinically important genetic changes may be missed by cytogenetic evaluation, even in sophisticated laboratories with a high rate of successful analysis. Some important genetic alterations are not identifiable by routine karyotyping. Examples are the *ETV6-AML1* fusion gene in B-lineage ALL and the *TAL1* rearrangements in T-cell ALL. Furthermore, molecular techniques are able to identify patients carrying a *BCR-ABL* rearrangement who do not show a Ph translocation in chromosome banding. Therefore, especially for those aberrations with a high prognostic impact, molecular screening should be considered if chromosome banding analysis fails or shows a normal karyotype *(382)*. Complementary techniques such as FISH and CGH must be used in selected cases if chromosome banding studies are not able to clarify the karyotype completely.

11.2. Implications of Chromosome Abnormalities on Prognosis

The implication of every prognostic factor on clinical outcome has to be analyzed in the background of the treatment strategies applied, because therapy has a major impact on the relevance of prognostic parameters. In terms of currently used standard protocols, t(12;21), dic(9;12), hyperdiploidy with a chromosome number between 51 and 55, and the t(10;14) in T-lineage ALL are associated with a favorable outcome. Patients with a normal karyotype, a deletion of the long arm of chromosome 6, or a 9p or 12p deletion show an intermediate prognosis. For patients with t(9;22), outcome is dismal even with intensive treatment strategies including allogeneic bone marrow transplantation. For patients with t(1;19), t(4;11), or t(8;14) and variants, prognosis could be improved with intensified therapy and risk-adapted treatment strategies *(268,318,373–375)*.

As therapy improves, the importance of various cytogenetic abnormalities in acute leukemia may change or diminish. There is evidence in childhood ALL that with more intensive chemotherapy, all patients fall into a single good-prognosis group, regardless of ploidy or specific karyotypic abnormalities. Even in the poor-prognostic subgroup of Ph-positive patients changes in treatment strategies may improve outcome. Recent studies in Ph-positive childhood ALL show that intensive chemotherapy improved outcome in a subgoup of patients *(283,350)*.

12. CONTROVERSIAL ISSUES

12.1. Which Standard Staining Techniques Should Be Applied to the Morphologic and Cytochemical Diagnosis of AML?

The diagnosis of AML is based first of all on morphologic features. This is because staining is quick and leads to a reliable diagnosis in most cases, with the exception of the subgroups AML M0 and AML M7. For all other catogeries, staining with MGG, MPO, and NSE (ANA or ANB) has to be performed on bone marrow aspirates and on blood films in parallel. Thus, cytomorphology will probably still be the backbone for diagnosis of AML in the next decade. Its value for the diagnosis of ALL may be restricted mainly to confirm the L3-type Burkitt cell leukemia in combination with immunophenotyping and cytogenetics. The role of cytomorphology for the definition of remission status has to be newly defined in comparison with other methods. Cytomorphology gives limited prognostic information.

12.2. Is the Updated AML Classification as Proposed by the WHO Useful?

This new proposal for the classification of AML follows a hierarchy beginning with cytogenetic entities and ending with cytomorphologic descriptions, as has been used in the FAB system. In addition, dysplastic features and the history of the AML with preceding MDS or chemotherapy is considered for classification subgroups.

The proposal has taken a major step toward classification based on biology. The new threshold for AML (>20% blasts) follows the same philosophy, because the biology of RAEB-T and AML were found to be the same. Also, the inclusion of AML after MDS and after preceding chemotherapy will help to clarify terminology and classification. With respect to the sub-

category of multilineage dysplasia, further prospective evaluations of its biologic relevance are urgently needed. For further studies, the FAB categories and the WHO classification should be investigated in parallel for clinical and biologic relevance.

In the next few years, the classification of acute leukemias will move further from morphology-based categories to subtypes that rely on immunophenotypic and mainly genetic characteristics. Standard cytogenetics will be replaced more and more by molecular methods, including microarray expression profiling and protein analysis. The main objective for the cytomorphologic and cytochemical approach will be to regulate and place all other techniques within an algorithm to make diagnosis quick, thus pointing the way to recognition of the leukemia as a biologic entity.

12.3. Which Cellular Antigens Have Proved Particularly Helpful in Lineage Assignment and Subclassification of Acute Leukemias?

Immunophenotyping studies have used different approaches to diagnose and subdivide the acute leukemias *(28,97,98)*. These include, first, the application of a general and comprehensive panel of MAb combinations; second, the use of a "minimal" screening panel for lineage assignment of the predominant blast population followed by a more thorough, secondary set of MAbs chosen according to the results obtained with the screening panel; and finally, the selection of a "targeted" approach based on morphologic, cytochemical, and clinical information. Obviously, the sequential or "targeted" approach is associated with some savings in reagent costs, but it requires more time and planning and depends on the availability of supportive additional information. Irrespective of the approach used, the MAb panel(s) should be sufficiently broad to allow lineage assignment, characterization of the definitive phenotype and maturational stage of the blast population, detection of aberrant antigen expression, and identification of normal cell populations possibly present in the specimen (e.g., normal mature T-cells or precursor B-cells). Over the past decade, the increasing availability of MAbs with specificity for hematopoietic precursors and differentiation antigens has improved our ability not only to recognize various subtypes of precursor B- and T-cell ALL, as well as AML, but also to characterize leukemic stem cells and to extend our knowledge about the hematopoietic stem-cell hierarchy that is susceptible to leukemic transformation *(2,383)*.

A flow chart representing the currently relevant antigenic targets in the immunophenotypic analysis of acute leukemias is shown in Fig. 1, and a brief description of these antigens and their cellular distribution is given in Table 7. These antigenic targets include lineage-specific markers for the lymphoid or myeloid differentiation, antigens mainly expressed by hematopoietic precursors, and antigens more closely associated with distinct maturational stages of myeloid and B- or T-cells. In this context, it is noteworthy that MAbs within CD groups recognize the same cellular antigen, but not necessarily an identical epitope, and thus may differ in their reactivity to leukemic blasts. Additionally, the directly fluorochrome-conjugated MAbs in dual- or multicolor immunofluorescence assays for the analysis of leukemia-associated antigens are associated with different thresholds for recognition of antigen expression. Therefore, published results in acute leukemias from different laboratories, especially regarding weakly expressed antigens, may not be readily comparable.

It should be noted that the most specific markers for the lymphoid or myeloid lineages are expressed in the cytoplasm at the immature stages of cell differentiation *(384–386)*, and their flow-cytometric detection requires appropriate permeabilization and fixation procedures *(387)*. However, in view of the coexpression of lineage-specific molecules such as CD79α in T-lymphoblastic leukemia/lymphoma *(388)* or acute myeloid leukemias *(389)* and MPO in ALL *(390,391)*, the importance of these markers for lineage assignment of leukemic blasts has to be confirmed in further clinical studies. The same applies to MAbs recognizing distinct epitopes of the human pre-B-cell receptor that may improve the precision of precursor B-cell ALL subclassification *(392)*.

Terminal deoxynucleotidyl transferase (TdT), initially considered as a specific marker of immature lymphoid cells, can be detected in AML with varying incidence, probably because of the application of techniques with different sensitivities (reviewed in ref. *393*). Hence, TdT may be used as a marker of hematopoietic immaturity or to corroborate the diagnosis of ALL in the presence of other lymphoid-specific antigens *(21)* but not as a lymphoid-specific antigen. In our experience, this quite comprehensive panel of MAbs, especially when used in combination with a multiparameter (three-color or more) flow-cytometric procedure, is suitable for answering the most relevant questions (see above) in diagnostic immunophenotyping and monitoring of acute leukemias. On the basis of these immunophenotypic analyses, a firm diagnosis of AML and ALL as well as a subclassification of precursor B- and T-cell ALL can be made in >98% of cases.

Although a detailed discussion of multiparameter flow cytometry is beyond the scope of this chapter, it should be mentioned that in view of its potential clinical utility, many attempts have been undertaken to standardize and validate laboratory procedures used in the immunophenotypic analysis, to select appropriate combinations of MAbs, and to standardize data analysis and interpretation, as well as data reporting *(24,28,30,97,98,394,395)*. The broad range of topics to be considered in this process illustrates the scope of the problem of achieving consensus. Thus, a major challenge for the future will be to improve the intra- and interlaboratory reproducibility of flow-cytometric immunophenotyping by performing stable, calibrated, and standardized measurements in such a way that identical neoplastic cells provide identical phenotypic patterns, whenever they are analyzed at different times and in different laboratories *(24)*. Hopefully, the recent consensus recommendations will contribute to achieving this goal by successfully implementing adequate flow-cytometric practices in hematopoietic malignancies.

Advances in immunophenotyping by using state-of-the-art flow cytometry and three or four fluorochromes conjugated with MAbs facilitate a multiparametric measurement of intrinsic and extrinsic cellular properties of leukemic cells *(23)*. From these studies, much more information as to the immunophenotypic characteristics of leukemic lymphoblasts

Table 7
Clusters of Differentiation (CD) Antigens Useful in the Diagnosis and Classification of AML and ALL

CD group	*Molecular and functional characteristics*	*Cellular reactivity within the lymphohematopoietic system*	*Comments on diagnostic value in leukemia diagnosis*
Myeloid lineage			
CD13	150-kDa type II membrane glycoprotein, homodimer, aminopeptidase N	Early committed progenitors of granulocytes and monocytes (CFU-GM) and maturing cells of these lineages	Expressed in most AML, coexpressed in 20–35% of ALL
CD14	55-kDa glycosylphosphatidylinositol-linked glycoprotein; LPS receptor	Mature monocytes (strong), macrophages, granulocytes (weak)	Expressed predominantly in mature myelomonocytic leukemias (AML-M4, M5b)
CD15	Carbohydrate, 3-FAL, X-hapten, Lewis-X (Le^X); adhesion molecule, ligand for E-, P-, L-selectin (CD15s)	Mature granulocytes and monocytes, myeloid and monocytic cells, Langerhans cells	Expressed in 50% of AML, aberrantly expressed in 5–10% of ALL, predominantly in pre-B-ALL with t(4;11)
CD33	67-kDa transmembrane protein, sialoadhesin	Myeloid and monocytic cells, early erythroblasts, megakaryoblasts	Expressed in most AML, coexpressed in 20–35% of ALL
CD36	88-kDa glycoprotein, platelet gpIIIb, gpIV	Megakaryocytes, platelets, mature monocytes and macrophages, erythroid precursors	Expressed predominantly in AML-M5, M6, M7
CD41	Platelet glycoprotein IIb, αIIb integrin chain, forms complex with CD61/β3 integrin chain	Megakaryocytes and platelets	Expressed in AML-M7
CD42b	Platelet glycoprotein Ibα, forms complex with CD41c (disulfide bond) and CD41a, CD41d; CD41a–d: receptor for vWf (von Willebrand factor)	Megakaryoctes and platelets, absence of CD42 complex leads to Bernard-Soulier syndrome	Expressed in AML-M7
CD61	Platelet glycoprotein IIIa, β3 integrin chain, forms complex with CD41/αIIβ integrin chain	Megakaryocytes and platelets	Expressed in AML M7
CD64	72-kDa glycoprotein, high-affinity IgG Fc receptor (FcγRI), receptor-mediated endocytosis of IgG-antigen complexes, antibody-dependent cellular cytotoxicity	Monocytes and macrophages, immature granulomonocytic progenitors, subset of dendritic cells, early myeloid lineages	Expressed in monoblastic/monocytic leukemia and in subsets of immature AML
CD65/CD65s	Carbohydrate, ceramide-dodecasaccharide/sialylated-CD65	Mature granulocytes, myeloid cells, monocytes	Expressed in most AML, aberrantly expressed in 5–10% of ALL, predominantly in pro-B-ALL with t(4;11)
CD66c	90-kDa GPI-linked glycoprotein, member of the carcinoembryonicantigen family	Mature granulocytes, myeloid cells, monocytes	Expressed by distinct subsets of B-cell precursor ALL (e.g., Ph^+, *ETV6-AML1*$^-$, and hyperdiploid cases)
T-lineage			
CD1a	49-kDa type I transmembrane glycoprotein, MHC I-like; binds to β_2-microglobulin; non-peptide antigen-presenting molecule	Cortical thymocytes, Langerhans cells	Defines cortical precursor T-cell ALL
CD2	50-kDa type I transmembrane glycoprotein, LFA-1; receptor for CD58 (LFA-3); adhesion and signal transducing molecule	Thymic and mature T-cells, most NK cells	Expressed in 70–85% of precursor T-cell ALL and approx. 10% of AML (especially M3 and M4Eo subtypes)
CD3	Complex of six polypeptide chains, component of the TCR (associated with TCRαβ or TCRγδ)	Thymic and mature T cells	Cytoplasmic expression defines precursor T-cell ALL, membrane expression in 25% of T-lineage ALL defines mature precursor T-cell ALL

Continued on next page

Table 7 (Continued)
Clusters of Differentiation (CD) Antigens Useful in the Diagnosis and Classification of AML and ALL

CD group	*Molecular and functional characteristics*	*Cellular reactivity within the lymphohematopoietic system*	*Comments on diagnostic value in leukemia diagnosis*
T-lineage (Cont.)			
CD4	55-kDa transmembrane glycoprotein, receptor for MHC class II ` molecules, receptor for HIV envelope glycoprotein (gp120)	Subset of thymocytes and mature T-cells (helper/inducer), monocytes, macrophages	Variably expressed by preT-, cortical, or mature precursor T-cell ALL, and AML (especially of monocytic origin)
CD5	67-kDa glycoprotein, scavenger receptor cysteine-rich (SRCR) family, costimulatory molecule and receptor for CD72	Thymic (weak expression) and mature (strong expression) T-cells,subset of mature B-cells	Expressed by 90–95% of precursor T-cell ALL
CD7	40-kDa glycoprotein,	T cells, NK cells, hematopoietic stem cells	Expressed in virtually all precursor T-cell ALL and approx. 15% of AML
CD8	32-kDa, αα homodimer or αβ heterodimer, coreceptor with TCR for MHC	Subset of thymocytes and mature T-cells (suppressor/cytotoxic), NK cells	Variably expressed by precursor T-cell ALL
CD group	Molecular and functional characteristics	Cellular reactivity within the lymphohematopoietic system	Comments on diagnostic value in leukemia diagnosis
B lineage			
CD19	95-kDa glycoprotein, associates with CD21; signal transduction	Expression from the earliest recognizable B-lineage cells to mature B-cells, follicular dendritic cells	Expressed in virtually all B-cell precursor ALL and a subset of AML [especially AML-M2 with t(8;21)]
CD20	33–37-kDa phosphoprotein, B-cell activation	B-cells	Expressed in 40% of B-cell precursor ALL
CD22	135-kDa type I glycoprotein, adhesion and signaling	Precursor and mature B-cells	Cytoplasmic expression in virtually all B-cell precursor ALL,membrane expression in B-cell precursor ALL and B-ALL
CD24	35–45-kDa glycosylphosphatidylinositol-linked glycoprotein	Precursor and mature B-cells, neutrophil granulocytes	Expressed in >90% of B-cell precursor ALL and some AML
CD79α	40–65-kDa glycoprotein, associated with CD79α; component of B-cell antigen receptor	Precursor and mature B-cells, plasma cells	Expressed in virtually all B-cell precursor ALL and aberrantly in some AML
NK			
CD56	175–220-kDa glycoprotein, neural cell adhesion molecule (NCAM), homotypic and heterotypic cell adhesion (in neural development)	NK cells, subset of T-cells	Expressed in some AML with t(8;21), t(15;17), acute monocytic leukemia and NK cell neoplasms
Non-lineage-associated			
CD10	100-kDa glycoprotein, zinc metalloprotease, neutral endopeptidase, common ALL antigen (CALLA)	Lymphoid precursors, germinal center B-cells, mature neutrophil granulocytes	Defines common ALL, expressed in approx. 40% of T-cell precursor ALL
CD34	105–120-kDa type I transmembrane glycoprotein, cell adhesion	Early lymphohematopoietic stem and progenitor cells	Expressed in 60–70% of B-cell precursor ALL, <10% of T-cell precursor ALL, and 40–50% of AML
CD45	180–220-kDa glycoprotein, leukocyte common antigen (LCA), tyrosine phosphatase, T- and B-cell antigen receptor-mediated activation	Expressed, typically at high levels, on all hematopoietic cells	Expressed in 90% of all B-cell precursor ALL, nearly all T-cell precursor ALL, and nearly all AML
CD117	145-kDa glycoprotein, tyrosine kinase receptor type 3, *c-kit*, stem cell factor (SCF) receptor	Hemopoietic stem and progenitor cells	Expressed in approx. 85–90% of B-cell precursor ALL and virtually all other subtypes of acute leukemia

Abbreviations: LFA, leukocyte factor-associated antigen; LPS, lipopolysaccharide; NK, natural killer; TCR, T-cell receptor.

and their functional features has emerged, which could be helpful in answering specific biologic as well as clinical questions; these data have not yet been applied on a large scale to routine immunophenotyping of ALL within clinical trials.

The enormous progress achieved over the last two decades in the identification of leukocyte surface antigens *(396)* has led to a deeper insight into the functional significance of various molecules that have proved useful in immunophenotyping of acute leukemias, as well as of novel molecules, more recently emerging as key regulators of cell proliferation, differentiation, adhesion, and induction of apoptosis. These novel markers, including costimulatory molecules *(397)* hematopoietic growth factor receptors *(398)*, adhesion molecules *(399)*, and chemokine receptors *(400)*, may hopefully complement the phenotypic classification in the future by providing a more clinically relevant functional subdivision of AML and ALL. In addition, considerable interest has focused on the expression of molecules mediating the multidrug resistance phenotype *(157)* and of proteins that play an important role in the regulation of drug-induced apoptosis in acute leukemias *(401)*. Preliminary results in both childhood and adult acute leukemias suggest that the expression of at least some of these molecules can be easily detected by flow cytometry and may provide clinically relevant information (e.g., Bcl-2-related proteins, death receptors for FasL and TRAIL, and caspases) *(158,161,402,403)*. However, further prospective investigations, preferably in the context of controlled clinical trials, are needed to clarify the importance of these molecules in the development of cytotoxic drug resistance in acute leukemia and to determine their prognostic significance compared with other parameters (e.g., in vitro drug sensitivity testing, early in vivo treatment response). These studies will hopefully also contribute to the elucidation of the precise mechanisms involved in the apoptotic killing of acute leukemia cells and of the strategies by which malignant cells escape killing by cytotoxic drugs *(401)*.

12.4. Should We Continue to Subdivide Precursor B- and T-Cell ALLs into Distinct Immunophenotypic Subgroups, and Which Subgroups Should be Identified?

Research over the past two decades, by applying immunophenotypic, cytogenetic, and molecular genetic techniques, has provided valuable information as to the diagnosis and classification of ALL. It has been demonstrated that precursor B- and T-cell ALL represents highly heterogeneous groups of malignancies that for the most appropriate tailoring of treatment strategies require a complex classification system, taking into account genotypic as well as immunophenotypic and clinical features (reviewed in ref. *348*). To be clinically useful, this classification system has to be easy, applicable to most patients in clinical trials, rapid to assess, reproducible, cost-effective, and capable of identifying biologically and clinically relevant entities.

In the past, criteria applied to define subgroups of precursor B- and T-cell ALL patients have differed markedly, and various immunophenotypic classifications of ALL have evolved, with most of them reflecting normal B- and T-cell ontogeny. The terms used to designate ALL immunophenotypic subgroups have been mainly based either on the presumptive B- and T-cell differentiation stages of normal lymphopoiesis, which define four to six subgroups of precursor B-cell ALL *(404,405)* and three to four subgroups of precursor T-cell ALL *(332,334,404,406)*, or on the expression of CD10, cytoplasmic or sIg, and different T-cell antigens, thus distinguishing broader categories of precursor B- and T-cell ALL *(341,384,407)*. More recently, the European Group for the Immunological Characterization of Leukemias (EGIL) has proposed guidelines for the immunophenotypic subclassification of B- and T-lineage ALL into different categories according to the degree of B- or T-lymphoid differentiation of the blast cells *(28)* (Table 8). Although conceptually, most of these models are useful in classifying ALL, several studies have shown that the immunophenotypic features of precursor B- and T-cell ALL and the normal stages of B- and T-lymphocyte development differ remarkably and that asynchronous or aberrant combinations of B- or T-cell-associated antigens, which do not reflect normal lymphoid ontogeny, have been demonstrated in the vast majority of precursor B- and T-cell ALL cases *(384,408–411)*. Based on these observations, it was suggested that leukemic blasts in precursor B- and T-cell ALL may not originate from phenotypically identical normal cellular counterparts or may result from the malignant proliferation of rare normal lymphoid cells not detectable with presently available methods *(408)*. Alternatively, asynchronous or aberrant expression of antigens in ALL has been postulated to reflect genetic alterations that occur during early stages of lymphoid differentiation (reviewed in ref. *412*).

In view of these data and only weak evidence in favor of basing treatment strategies on immunophenotyping studies, the clinical importance of subclassification into distinct precursor B- and T-cell subsets has been questioned *(348)*, and alternative criteria not representing a classification of ALL derived from an

Table 8
Classification of ALL

Category	*Definition*
B-lineage ALL[a]	**CD19+ and/or CD79α+ and/or CD22+**
Pro-B (B-I)	No expression of other B-cell differentiation antigens
Common (B-II)	CD10+
Pre-B (B-III)	Cytoplasmic IgM+
Mature B (B-IV)	Cytoplasmic or surface κ or λ+
T-lineage ALL[b]	**Cytoplasmic/membrane CD3+**
Pro-T (T-I)	CD7+
Pre-T (T-II)	CD2+ and/or CD5+ and/or CD8+
Cortical T (T-III)	CD1a+
Mature T (T-IV)	Membrane CD3+, CD1a-
α/β + (group a)	Anti-TCR α/β+
γ/δ + T-ALL (group b)	Anti-TCR γ/δ+
ALL with myeloid antigen expression (My + ALL)	

[a] Positive for at least two of the three markers. Most cases are terminal deoxynucleotidyl transferase (TdT)+, HLA-DR+ except for B-IV, which often is TdT–.

[b] Most cases are TdT+ HLA-DR-CD34–, but these markers are not considered for diagnosis or disease classification.

Table 9
Scoring System for Biphenotypic Acute Leukemias (BALs)[a]

Points	*B-lineage*	*T-lineage*	*Myeloid lineage*
2	CD79α cy IgM (cy) CD22	CD3 (cy/m) anti-TCR α/β anti-TCR γ/δ	MPO
1	CD19 CD10 CD20	CD2 CD5 CD8 CD10	CD13 CD33 CD65s CD117
0.5	TdT CD24	TdT CD7 CD1a	CD14 CD15 CD64

Abbreviations: cy, cytoplasmic; m, membrane; TCR, T-cell receptor; MPO, myeloperoxidase; Tdt, terminal deoxynucleotidyl transferase.

[a]Total scores must exceed 2 for the myeloid lineage and 1 for the lymphoid lineages. The value of each marker is given in the far left column.

ontologic model have been proposed *(413)*. However, there is now a great deal of evidence that several immunophenotypic subgroups of precursor B- and T-cell ALL have innate cell biologic and clinical features that should be taken into consideration when interpreting treatment outcome. Moreover, intrinsic pharmacologic differences in responsiveness to cytotoxic drugs, recently described in immunophenotypic subgroups, may contribute to devising truly lineage- and/or subgroup-specific therapeutic interventions for patients with ALL *(414,415)*.

In precursor B-cell ALL, the following subclassification may provide the most useful information to guide treatment selection: (1) pro-B ALL, frequently associated with 11q23 rearrangements in both childhood and adult ALL; (2) common and pre-B ALL, with both subsets harboring a high degree of genotypic diversity [e.g., Ph translocation, t(12;21), t(1;19), hyperdiploidy]; and probably (3) transitional pre-B ALL or late pre-B ALL, characterized by leukemic blasts that express both cytoplasmic μ and sIg μ heavy chains without κ or λ light chains, and show an excellent response to precursor B-cell-directed treatment *(416)*. Distinctions such as that between pro-B and common/pre-B ALL are especially helpful in patients in whom karyotyping has not been possible, since it may direct molecular genetic studies for gene rearrangements (e.g., *MLL* or *BCR-ABL*).

In precursor T-cell ALL, no clear-cut correlations of immunophenotypic subgroups with genetic abnormalities have yet been established. However, recent studies suggest that immature subgroups with a pro-T and pre-T phenotype differ markedly with respect to their cell biologic features and treatment response from the predominant subgroup, which has a CD1a-positive phenotype *(310,320,342)*, thus corroborating the value of immunophenotypic subclassification of precursor T-cell ALL. The relationships of other precursor T-cell ALL subgroups (i.e., pro-T vs pre-T, αβ- vs γδ-positive ALL) with cell biologic features and treatment outcome are still unclear and have to be prospectively evaluated within controlled clinical trials in both childhood and adult ALL.

12.5. Which Criteria Should Be Used to Establish Aberrant Antigen Expression and to Classify Biphenotypic Acute Leukemia?

The widespread application of flow-cytometric immunophenotyping with a large panel of MAbs to myeloid- and lymphoid-associated differentiation antigens has led to the recognition of acute leukemias with blasts coexpressing antigens associated with different lineages. Different hypotheses have been postulated to explain the origin of these acute leukemias, which often display heterogeneous cell biologic features, leading to the concept of "lineage infidelity" *(95)*, which implies aberrant phenotypic features frequently resulting from specific genetic alterations, and the theory of "lineage promiscuity" *(417)*, whereby the malignant transformation occurs in a progenitor cell with the capacity for both myeloid and lymphoid differentiation. Unfortunately, much controversy has surrounded the criteria for identifying such leukemias, and a variety of terms have been used to refer to these acute leukemias, such as hybrid, biphenotypic, mixed-lineage, myeloid antigen-positive ALL (My+ ALL), and lymphoid antigen-positive AML (Ly+ AML), thus causing considerable confusion and complicating the assessment of the clinical importance of these observations.

More recently, strict and well-defined criteria have been proposed that were aimed at distinguishing biphenotypic acute leukemia (BAL), also referred to as "true" mixed-lineage leukemia, from those cases with aberrant expression of one or more markers from another lineage (e.g., My+ ALL and Ly+ AML) *(25,28,340,418)*. Criteria and scoring systems applied to the diagnosis of BAL are based on the number and degree of specificity of the markers (lymphoid and myeloid) expressed by the leukemic blasts and have been described in detail elsewhere *(25,28,340)*. The diagnosis of both BAL (Table 9) and My+ ALL or Ly+ AML requires multiparametric flow cytometry with at least two fluorochromes conjugated to different MAbs to demonstrate coexpression of lineage-specific (e.g., MPO, CD22, CD79α, CD3) and/or lineage-associated antigens. Other markers with a high degree of specificity (e.g., MAbs recognizing surrogate light-chain components) *(392)* will be incorporated into these scoring systems in the near future and hopefully improve its reliability. Although extensive data on the cell biologic features and response to treatment of BAL or mixed-lineage leukemia are not yet available, preliminary results suggest that they represent an uncommon subtype with distinct genetic (e.g., Ph translocation, 11q23 rearrangements, complex cytogenetic abnormalities) and clinical features as well as a poor prognosis *(418–422)*. Based on immunophenotyping, cytogenetic and molecular genetic findings, and the documented phenomenon of in vivo as well as in vitro phenotypic switches in some cases of BAL, it has been suggested that these leukemias arise in a multipotent progenitor cell with the capability of differentiating along both myeloid and lymphoid lineages *(421)*.

In contrast to BAL, My+ ALL and Ly+ AML occur frequently. Their incidence has varied considerably among independent studies, both overall and with regard to individual antigens (reviewed in refs. *88* and *412*), ranging from 5 to >50% for My+ ALL and from 10 to 30% for Ly+ AML. This wide variability has been attributed to a number of causes, including

the lack of consistent criteria for the diagnosis of My+ ALL or Ly+ AML and for defining positive results, the utilization of various panels of MAbs, the lack of lineage specificity of most of the MAbs used, and several technical factors (e.g., distinct sensitivities of fluorochromes and flow cytometers, inconsistent gating strategies) *(88,282,412,419)*. In view of previous studies pointing to myeloid-antigen expression as a predictor of poor prognosis in both childhood and adult ALL *(423,424)*, considerable interest has focused on the cell biologic features and clinical significance of this subgroup of acute leukemias. Several recent studies, including more than 4000 pediatric patients with ALL *(425–427)* and our own data in almost 5000 children treated within the ALL-BFM 86, 90, and 95 trials *(320,327,428)*, have failed to demonstrate an association of My+ ALL with poor outcome. In some of these studies, myeloid-associated antigen expression was clearly associated with certain genetic features of leukemic cells, particularly *MLL* and *ETV6-AML1* rearrangements *(293,427–429)*. In contrast to childhood My+ ALL, the clinical importance of myeloid-associated antigen expression in adult ALL is still unknown. The presence of myeloid-associated antigens has been associated with a poor outcome in some *(423,430)* but not all studies *(270,431,432)*. Most of these studies, however, included only a relatively small number of patients, have not always carefully excluded minimally differentiated AML (AML M0) *(423)*, and, most important, have not adequately taken into account the prognostic importance of specific genetic abnormalities frequently found in adult patients with My+ ALL, such as Ph positivity or 11q23 rearrangements *(268,270,282,433)*. Further prospective studies, consistently based on well-defined diagnostic criteria, are urgently needed to elucidate more accurately the biologic heterogeneity of My+ ALL and to establish its clinical relevance in adult patients.

A critical review of data published in the literature revealed that most retrospective and prospective studies failed to demonstrate any prognostic significance for Ly+ AML, except for CD7+ AML *(88)*. The latter subgroup has been associated with more frequent expression of progenitor-associated markers (e.g., CD34, CD117, HLA-DR, TdT), concomitant rearrangements of *Ig* and/or *TCR* gene rearrangements, and poor prognosis in most (but not all) studies in both childhood and adult AML *(86,87,162,434–437)*. It should be noted that immature CD7+ AML and pro-/pre-T ALL occasionally show biologic similarities, such as reactivity with MAbs recognizing antigens expressed on both immature T-cell ALL and AML *(438)*, responsiveness to several growth factors *(437)*, expression of *c-kit* at the mRNA and protein levels *(164,165,340,439)*, expression of the multidrug resistance phenotype *(440)*, and similar *TCR*δ gene rearrangements *(441)*, suggesting that in at least some CD7+ acute leukemias, malignant transformation has arisen in a pluripotent progenitor cell with variable differentiation potential along both myeloid and T-lymphoid lineages *(429)*.

Given the significant associations between expression of several lymphoid-associated antigens by AML and specific genetic abnormalities, such as CD19 in AML with t(8;21) and CD2 in AML with t(15;17), as well as AML with inv(16) or t(16;16), cytogenetic and molecular data have to be incorporated into the classification of Ly+ AML, and future studies evaluating the prognostic significance of Ly+ AML have to take into consideration its genetic background.

12.6. Which Conventional and Molecular Cytogenetic Techniques are Necessary to Identify Cytogenetic Alterations and to Provide Cytogenetic Information that Has Clinical Relevance?

In general, classical cytogenetics using banding techniques is still the gold standard for the genetic classification of acute leukemias. These techniques should be performed in each patient with acute leukemia at diagnosis as well as at relapse. New techniques such as FISH, Southern blot, and PCR analyses have added important information to the more sophisticated subgrouping of acute leukemia. These techniques should be used on demand in cases in which these investigations can give information that cannot be obtained by banding analysis. Screening with these techniques for each detectable genetic aberration is very expensive and labor-intensive and thus not cost-effective. In childhood B-cell precursor ALL, screening with RT-PCR or FISH for the detection *ETV6-AML1* rearrangement seems mandatory, because t(12;21) is mostly not detectable with conventional cytogenetics. Furthermore, in cases of T-cell precursor ALL, checking for *TAL1* rearrangements with Southern blotting or RT-PCR should be performed. If no banding analysis is available, screening for the most important abnormalities regarding prognosis is recommended: in B-cell precursor ALL: *BCR-ABL* rearrangement, *E2A-PBX1* rearrangment, *MLL* rearrangements, hyperdiploidy, 9p deletions; in mature B-ALL: translocations involving *MYC*; in precursor T-cell ALL: *TAL1* rearrangement and in AML: rearrangements of *AML1-ETO*, *PML-RAR*α, *CBF*β*-MYH11*, *MLL* rearrangements, monosomies 5/7, and deletions 5q/7q and 17p.

12.7. Do We Need an International Cytogenetic Classification for Acute Leukemias and Which Aspects are Important?

An international cytogenetic classification is urgently needed to allow a comparison among different studies. On the one hand, a hierarchical classification according to primary chromosome aberrations is needed; on the other hand, for clinical use a prognostic grouping for distinct cytogenetic abnormalities is required. This is problematic because the prognosis of cytogenetic subgroups is influenced by therapy. Therefore, a biologically orientated classification is necessary that will allow analysis of the impact of certain treatments on a cytogentically defined subgroup of patients.

In AML, the favorable cytogenetic subgroup is well defined, and nearly all study groups agree that patients with t(15;17), t(8;21), or inv(16)/t(16;16) belong to this subgroup. The impact of additional abnormalities on favorable aberrations has to be determined in metaanalyses. For the intermediate and unfavorable subgroups, discrepancies occur. The intermediate subgroup is a mixture of patients with normal karyotypes, karyotypic abnormalities with proven intermediate prognosis, and karyotypic aberration of unknown prognostic significance owing to low frequency of these aberrations. For future analysis, more informative results can be obtained if the intermediate group is analyzed in three subgroups, as mentioned above. In patients

with a normal karyotype, molecular studies will help to define distinct entities within this probably heterogeneous group. Metaanalyses will help to clarify the prognostic impact of rare abnormalities of as yet unknown prognostic impact. Concerning karyotypic abnormalities assigned to the unfavorable group, the definition of complex aberrant karyotypes in particular has to be standardized and based on biologic features, rather than on the number of abnormalities. Further molecular studies in this subgroup may clarify the underlying biologic mechanism leading to genomic instability and a poor prognosis.

Cytogenetic classifications for clinical use based on prognostic impact will change with the implementation of new treatment strategies. Therefore, reports on the prognostic impact of distinct cytogenetic abnormalities must always be evaluated in the background of the applied treatment.

12.8. Do We Need Cytogenetic Analyses in Relapsed AML?

Only a few studies on the cytogenetics of relapsed AML have been published. These studies and our own data, however, show that karyotype is a prognostic factor in AML at relapse. From 50 to 60% of patients with a favorable or intermediate karyotype in relapse achieve a complete remission, contrasted with only 20% of cases with unfavorable cytogenetics *(442)*. Relapse occurs later in patients with a favorable or intermediate karyotype than in patients with unfavorable cytogenetics *(443)*. Compared with cytogenetics at diagnosis, the same karyotype is found in relapse in 40–50% (20–30% show normal karyotypes and about 20% the same aberrant karyotype at diagnosis and in relapse). From 15 to 20% of patients show a normal karyotype at diagnosis and an aberrant one at relapse, whereas 25% of those who had an aberrant karyotype at diagnosis gain additional chromosomal aberrations at relapse. In 2–5%, a new clone is observed at relapse that is unrelated to the clone observed at diagnosis *(179,180)*. In these patients, secondary AML rather than a relapse of the primary AML should be suspected.

12.9. What Is the Impact of Cytogenetics on Therapy-Related AML?

Leukemia secondary to chemotherapy accounts for 10–15% of all AML cases. The therapy-related (t-)AMLs are usually divided into two subgroups, depending on whether the patient has received alkylating agents or drugs targeting topoisomerase II. t-AMLs related to alkylating agents are frequently characterized by a preceding myelodysplastic phase, a long interval between cytotoxic treatment and the appearance of t-AML (36–72 mo), cytogenetic abnormalities involving chromosomes 5 and 7, and often complex aberrant karyotypes and a poor response to therapy. t-AML related to therapy with topoisomerase II inhibitors usually presents as overt leukemia without a myelodysplastic phase with either an M4 or an M5 subtype according to FAB classification, has a short latency period (12–36 mo), and shows balanced chromosome aberrations, primarily translocations involving chromosome bands 11q23 and 21q22 and a more favorable response to chemotherapy *(218,444–448)*. Translocations involving 11q23 predominate following therapy with epipodophyllotoxins, whereas patients with translocations to 21q22, inv(16), and t(15;17) most often have received anthracyclines. However, a multivariate analysis in a large series of patients with t-AML and balanced translocations showed that younger age and not a specific type of DNA topoisomerase II inhibitor seems to predispose to the development of t-AML with 11q23 translocations *(449)*. In accord with these data, our own findings indicate that patients with balanced chromosomal aberrations such as t(8;21), inv(16), t(15;17), or t(11q23) were significantly younger than those with other abnormalities (median age 45 vs 60 yr) and showed a shorter latency period between the primary tumor and t-AML (30 vs. 81 mo) *(450)*.

Compared with *de novo* AML, t-AML has a higher incidence of clonal chromosome abnormalities. In 75–96% of cases, karyotypic aberrations are detected *(226,450–452)*. The spectrum of such changes is comparable, but the distribution varies, as 11q23 abnormalities and complex aberrant karyotypes occur more often in patients with t-AML than in *de novo* AML *(447,450,453,454)*.

Overall, t-AML responds less well to treatment than does its *de novo* counterparts. Recent data show that, as in *de novo* AML, cytogenetics are an important prognostic factor in t-AML; moreover, if corresponding cytogenetic subgroups are compared according to response, outcome does not differ much *(450,451,454–456)*.

Work is in progress to identify parameters that predispose patients to the development of t-AML. Defects in DNA repair mechanisms as well as polymorphisms in enzymes involved in the metabolism of antileukemia drugs are under investigation *(457,458)*. However, as t-AML is a secondary and in some cases a tertiary neoplasm, and in a quite high proportion of patients surgery was the only treatment for the primary tumor, a predisposition of cancer patients to leukemia, independent of previous chemo- or radiotherapy, cannot be excluded *(444)*.

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2 Classification of Acute Leukemias

Perspective 2

FREDERICK G. BEHM

CONTENTS

1. INTRODUCTION

A useful classification of acute leukemia must be reproducible, must impart understanding of leukemogenesis and clinical behavior, and (most importantly) be clinically relevant, which makes such a classification indispensable for designing and comparing clinical trials. Classification systems by their very nature may influence the design of treatment regimens and may even bias investigations of leukemogenesis. The discovery over 30 years ago that the acute lymphoid and acute myeloid leukemias differed in their responses to chemotherapeutic agents set the stage for the development of clinically useful classifications. The first of these was based on the morphologic features of leukemic blasts, with different groups of hematologists establishing their own terminology and diagnostic criteria. This lack of uniform nomenclature and hence comparable classifications posed a major obstacle to rapid progress in the treatment and understanding of leukemia pathobiology.

The first internationally accepted classification of acute leukemia was proposed in 1976 by the French–American–British (FAB) Cooperative Group *(1)*. The initial FAB classification was based solely on morphologic criteria that were subsequently refined in 1981 and 1985 *(2–4)*. Unfortunately, the revisions largely ignored exciting immunologic and cytogenetic discoveries that were contributing to an improved understanding and better treatment designs in acute leukemia. The morphologic, immunologic, and cytogenetic (MIC) classification, introduced in 1988, was the first to recognize the usefulness of cytogenetics for identifying subgroups of acute leukemia *(5,6)*. The MIC system recognized additional subgroups of acute leukemia not discernible in the FAB classification. Modifications of the FAB classification were also recommended by a National Cancer Institute-sponsored workshop in 1990 *(7)*.

Treatment of pediatric leukemia is one of the great successes of modern cancer therapy. Much of the success can be attributed to the recognition of important patient risk factors and the design of effective therapy for patients at high risk of treatment failure *(8)*. Although they are still important, older risk factors based on a patient's physical manifestations or hematologic and biochemical testing have been largely replaced by biologic features of the leukemic cell. Present risk assignments depend heavily on combined morphologic, immunologic, cytogenetic, and (more recently) molecular genetic studies. The recently introduced World Health Organization (WHO) classification takes into account morphologic and immunologic features plus well-studied, common nonrandom chromosomal abnormalities that clearly influence the laboratory and clinical features of

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acute leukemia *(9)*. No doubt current and future gene profiling studies and in-depth studies of gene function, together with a better understanding of host factors and responses to pharmacologic agents, will result in more functionally useful classifications. To paraphrase an old cliché, revised classifications of acute leukemia are almost as certain as new taxes and death. This chapter does not attempt to reiterate the laboratory, biologic, and clinical features of every recognizable type of acute leukemia, as these are available from other sources *(10–12)*, including the preceding chapter in this book, rather, it seeks to highlight the major advances, limitations, and controversies of past and current classifications.

2. DEFINITIONS OF ACUTE LEUKEMIA

Examination of the bone marrow is required by FAB criteria to make a diagnosis of acute leukemia. Thus, acute leukemia is established when at least 30% of the total nonerythroid cells in the marrow are blasts or have features of acute promyelocytic leukemia *(3)*. However, examples of leukemia are encountered in which the blasts comprise >30% of the leukocytes in peripheral blood but less than that in bone marrow. Others recommend that the diagnosis of acute leukemia be accepted when the blast percentage in the peripheral blood of bone marrow is >30% *(13)*. The WHO classification uses >20% blasts in the marrow or peripheral blood as a diagnostic criterion *(9)*.

The definition of a "blast" would appear to be straightforward, but this is often difficult to apply in practice. The FAB group recognized three types of leukemic blasts: lymphoblast, a cell with a high nuclear/cytoplasmic (N/C) ratio, indistinct-to-prominent nucleoli, and the absence of detectable myeloperoxidase by cytochemical staining; type I myeloblast, an agranular cell with a high N/C ratio, uncondensed chromatin, and prominent nucleoli; and type II blast, a cell with type I myeloblast features but containing a few azurophilc granules *(3)*. With the intention of improving interobserver agreement, the 1990 National Cancer Institute Workshop recommended additional leukemic cell types for defining myeloblastic leukemia *(7)*. These included type III myeloblasts, which contained more granules than type II myeloblasts, promyelocytes of acute promyelocytic leukemia, monoblasts and promonocytes of monocytic leukemias, and megakaryoblasts of acute megakaryoblastic leukemia. While these proposals appear to be useful, agreement among observers as to what constitutes a leukemic blast in a given bone marrow specimen often remains an unspoken problem.

The term *acute lymphoblastic leukemia* (ALL) is universally accepted and conveys a clear understanding of the type of leukemia being considered. However, the designation *acute myeloid leukemia* (AML) can be confusing to those outside the hematology–oncology community and sometimes to those within the community. Acute myeloid leukemia, in the strictest sense, refers to malignancies of myelocytic or granulocytic origin. However, in the FAB classification and common usage, this term is applied to leukemias of myeloid, monocytic, erythroid, or megakaryocytic origin. *Acute nonlymphoid leukemia*, a poor substitute for describing all leukemias not of lymphoid lineage, lost favor with hematologists but still creeps into books and journal articles *(7)*.

3. FAB CLASSIFICATION: ACCOMPLISHMENTS AND LIMITATIONS

In 1976, the first FAB Cooperative Group proposal recognized three major hematologic malignancies—acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and myelodysplastic syndrome (MDS)—and proposed subgroups within each of these malignant processes *(1)*. The expectation of the FAB group was that improved uniformity of classification would lead to the identification of clinically relevant subtypes. The lymphoblastic leukemias were divided into three subtypes based on the cytologic features of the leukemic blasts. Initial studies showed that the ALL-L3 subtype was associated with a mature B-phenotype and the t(8;14) chromosomal translocation; however, no similar correlation or reproducible clinical outcomes could be attributed to the L1 and L2 subtypes. Hence, the FAB group revised their classification of ALL in 1981 *(2)*. Subsequent multivariant analysis of clinical studies showed no additional prognostic information beyond that obtained from leukocyte count, age, race, and karyotype ploidy *(14)*. No sooner had the revised FAB classification been proposed than immunologic investigations of ALL showed the importance of differentiating B- from T-lineage ALL and recognizing expression of the common ALL antigen (CALLA) *(14–18)*. The introduction of monoclonal antibody technology led to the development of reagents that opened an era of extensive investigation producing newer and better classifications of ALL based on cell lineage and differentiation rather than morphology alone.

Electron microscopic investigations and rudimentary immunophenotyping studies of the 1970s and early 1980s were not lost on the authors of the FAB classification. By 1985, the FAB group had revised their AML classification and proposed a new AML subtype, acute megakaryoblastic leukemia (AML M7) *(4)*. Morphologic and numerical criteria for AML and MDS subgroups were revised in response to criticisms of the original 1976 proposal, and several years later, criteria were proposed for diagnosing myeloperoxidase-negative myeloid leukemia (AML M0) *(19)*. The FAB group offered no revision of their earlier classifications of lymphoblastic leukemia; indeed, investigators using immunophenotyping were busy proposing new classifications of ALL based on lineage and stage of lymphocyte differentiation. The descriptions of nonrandom cytogenetic abnormalities associated with distinct subgroups of acute leukemia were just making their way to press and thus were not incorporated in the revised FAB classification. The revised classification of AML was not without its critics. Problems identified with the revisions included definitions of what constitutes a leukemic blast, the distinction between acute leukemia and myelodysplastic syndrome based on blast numbers, the use of lysozyme determinations for separating some cases of myeloid from monocytic leukemias, and, importantly, the lack of studies to substantiate that the revisions were clinically useful *(20)*. As discussed later, similar criticisms apply in part to the recently proposed WHO classification.

The FAB classification can be credited with providing the first uniform approach to the classification of acute leukemias, with resultant improvement in separations of ALL, AML, and MDS. More important, this classification facilitated comparisons among treatment protocols by better defining the fre-

Table 1
Morphologic, Immunologic, and Cytogenetic (MIC) Classification of Acute Leukemia[a]

		Immunologic markers							
MIC group	*FAB*	*CD2*	*CD7*	*CD10*	*CD19*	*TdT*	*cIg*	*cIg*	*Karyotype*
Acute lymphoblastic leukemia (ALL)									
Early B-precursor ALL	L1, L2		–	+	+	+	–	–	t(4;11); t(9;22)
Common ALL	L1, L2		–	+	+	+	–	–	6q–; near-haploid; del(12p), or t(9;22)
Pre-B ALL	L1		–	+	+	+	+	–	t(1;19), t(9;22)
B-cell ALL	L3		–	+/–	+	–	–	–	t(8;14); t(2;8); t(8;22)
Early T-precursor ALL	L1, L2	+	+		–	+			t/del(9p)
T-cell ALL	L1, L2	+	+		–	+			6q–

		Immunologic markers						
MIC group	*FAB*	*CD7*	*CD19*	*CD13*	*CD33*	*GPA*	*CD41*	*Karyotype*
Acute myeloid leukemia (AML)								
M2/t(8;21)	M2	–	–	+	+	–	–	t(8;21)(q22;q22)
M3/t(15;17)	M3,M3v	–	–	+	+	–	–	t(15;17)(q22;q12)
M5a/del(11q23)	M5a (M5b,M4)	–	–	+	+	–	–	t/del(11)(q23)
M4Eo/inv(16)	M4Eo	–	–	+	+	–	–	del/inv(16)(q23)
M1/t(9;22)	M1 (M2)	–	–	+	+	–	–	t(9;22)(q34;q11)
M2/t(6;9)	M2 or M4 with basophilia	–	–	+	+	–	–	t(6;9)(p21-22;q34)
M1/inv(3)	M1 (M2, M4, M7) with thrombocytosis	–	–	+	+	–	–	inv(3)(q21q26)
M5b/t(8;16)	M5b with phagocytosis	–	–	+	+	–	–	t(8;16)(p11;p13)
M2 Baso/t(12p)	M2 with basophilia	–	–	+	+	–	–	t/del(12)(p11-13)
M4/+4	M4 (M2)	–	–	+	+	–	–	+4

+, positive; –, negative; no symbol, not specified by MIC workshop.
Abbreviations: FAB, French–American–British Classification; TdT, terminal deoxynucleotidyl transferase; GPA, glycophorin A.
[a] Data from refs. *5* and *6*.

quency and types of leukemias among patients entered on these protocols. Indeed, AML treatment protocols of several cooperative study groups currently incorporate the FAB classification for purposes of patient stratification. Even the findings of current immunologic, cytogenetic, and molecular studies of acute leukemias are frequently put into prospective according to the FAB subtypes studied. However, the limitations of the FAB classification are now obvious. First, the reproducibility of the morphologic separation of the different AML subtypes in various studies ranges from 60 to 90%. Second, immunologic and genetic investigations of the past decade have identified leukemic subtypes not discernible by FAB criteria. Third, FAB criteria for separating MDS from AML are not practical and easily reproducible. Fourth, the FAB classification does not identify patients whose leukemias arise out of a background of MDS and thus may relegate patients to less-than-optimal treatment approaches.

4. MIC COOPERATIVE GROUP CLASSIFICATION OF ACUTE LEUKEMIA

In 1986, the First MIC Cooperative Study Group published its morphologic, immunologic, and cytogenetic criteria for the classification of ALL *(5)*. Shortly thereafter the second workshop of the MIC group was held to promote similar criteria for the classification of AML *(6)*. The publications of both workshops built on morphologic criteria of the 1985 revised FAB classification. The recommendations of the workshops included retention of the FAB criteria for ALL and AML subgroups L1–L3 and M1–M7, respectively, with no major changes except for recognition of an M2 Baso subgroup. This subtype was applied to M2 leukemias that had evidence of basophilic granules in blast cells and maturing granulocytes. The MIC group also proposed panels of antibodies to B-, T-, erythroid-, megakaryocytic-, and myeloid lineage-associated antigens and immunologic techniques to be used in studying acute leukemias. The second MIC workshop was the first to recommend that bilineage and biphenotypic leukemias be classified as unique categories and stated the importance of recognizing these subtypes in therapeutic trials to establish their laboratory features and clinical significance.

The major emphasis of these workshops was recognition of the increasingly important role played by cytogenetics in the characterization of leukemia. The MIC classification proposed six subtypes of ALL and 10 subtypes of AML that are characterized by unique morphologic, immunologic, and cytogenetic features (Table 1) *(5,6)*. Another 10 karyotypic AML groups (+8, –7, 7q–, 5q–, –Y, +21, 9q–, 17q, 20q–, and +22) without specific morphologic or immunophenotype associations were also proposed. In comparison with the revised FAB classification, the MIC classification was insightful in recognizing the important role that cytogenetics would play in the treatment of acute leukemias. On the downside, the MIC classification was applicable to only 50% of patients with AML. Unfortunately, one-half of patients with AML would not have a karyotypic

Table 2
Immunologic Classification of Acute Leukemias

SJCRH classification (21)		*EGIL classification (90)*	
Immunologic subgroup	*Immunophenotypic profile*	*Immunologic subgroup*	*Immunophenotypic profile*
B-lineage ALL	CD19+/CD22+/cyCD3–/MPO–	B-lineage ALL	CD19+ and/or CD79α+ and/or CD22+
Early pre-B	CD79α±/CD10+/Igμ–	B-I (pro-B)	No B-cell differentiation antigens
		B-II (common B)	CD10+
Pre-B ALL	CD79α+/CD10+/cyIgμ+	B-III (pre-B)	cyIgμ+
Transitional (late) pre-B	CD79α+/CD10+/cyIgμ+/sIgμ+		
Mature B	CD79α+/CD10±/cyIgμ+/sIgμ+/sIgλ+or sIgκ+	B-IV (mature B)	cyIg or sIg κ+ or λ+
T-lineage ALL	CD7+/cyCD3+/CD22–/CD79a±/MPO–	T-lineage ALL	Cytoplasmic/surface CD3+
Pre-T	sCD3–/CD5–/CD1–/CD4–/CD8–/CD10–	T-I (pro-T)	CD7+
Early-T	sCD3–/CD5+/CD1–/CD4–/CD8±/CD10–	T-II (pre-T)	CD2+ and/or CD5+ and/or CD8+
Common-T	$sCD3^{lo}$/CD5+/CD1±/CD4±/CD8±/CD10±	T-III (cortical T)	CD1a+
Late-T	$sCD3^{hi}$/CD5+/CD1–/CD4+ or CD8+/CD10–	T-IV (mature T)	Surface CD3+, CD1a–
		α/β (group a)	TCR α/β+
		γ/δ (group b)	TCR γ/δ+
Early myeloid (AML-M0)	Anti-MPO± but enzymatic MPO-/CD33±/ CD13±/CD15±/CD117±/CD61–/GPA–	Early myeloid (AML-M0)	MPO± but enzymatic MPO–/CD13+/CD33+/ CD65+/and-or CD117+
Myeloid lineage	CD34±/HLA-DR±/MPO±/CD33±/CD13±/ CD15±/CD64- or wk/CD117±/CD61-/GPA-	Myelo/monocytic lineage	MPO+/CD13+/CD33+/CD65+/and-or CD117+
Monocytic lineage	CD34–/HLA-DR+/MPO±/CD33/CD13±/ CD14±/CD15±/CD64+/CD117±/CD61–/GPA–		
Megakaryocytic lineage	CD34±/HLA-DR±/MPO–/CD33±/CD13±/ CD15±/CD64–/CD117±/CD61+/GPA–	Megakaryocytic lineage	CD41+ and/or CD61+ (surface or cytoplasmic)
Erythroid (pure) lineage	CD34±/HLA-DR±/MPO–/CD33±/CD13±/ CD15±/CD64-/CD117±/CD61-/GPA+	Erythroid lineage	Early/immature: unclassified by markers Late/mature: GPA+
Undifferentiated	CD45+/CD34±/CD19±/CD22–/CD79α–/ cyCD3-/CD7±/CD5–/MPO-/CD33±/ CD13-/CD15-/CD117±/CD61–/GPA–	Undifferentiated	Often CD34+/HLA-DR+/CD38+/CD7+

Abbreviations: SJCRH, St. Jude Children's Research Hospital; EGIL, European Group for the Immunological Characterization of Leukemia; MPO, myeloperoxidase; cyIg, cytoplasmic immunoglobulin; sIg, suface immunoglobulin; TCR, T-cell receptor.

Table 3
Correlation of Cytogenetic Abnormalities with Leukocyte Antigen Expression Profiles

Karyotype	*Genes involved*	*Leukemia subtype*	*Leukocyte antigen profile*
t(4:11)(q21;q23)	*AF4*, *MLL*	Early pre-B-ALL	CD45+/CD34+/CD19+/CD24– or wk/CD10– or wk/CD15+
t(12;21)(p12;q22)	*TEL*, *AML1*	Early pre-B- or pre-B-ALL	CD45+/CD34±/CD19+/CD24+/CD10+/CD9– or wk/CD13±/CD33±
t(1;19)(q23;p13)	*PBX1*, *E2A*	Pre-B-ALL	CD45+/CD34–/CD19+/CD24+/CD10+/CD15+/cyIgμ+/sIgμ±
t(9;22)(q34;q11)	*ABL*, *BCR*	Early pre-B- or pre-B-ALL	CD45+/CD34±/CD10+/CD24+/CD9+/CD13±/CD33±
t(8;21)(q22;q22)	*ETO*, *AML1*	AML-M2 (some M1 or M4)	CD45+/CD34+/HLA-DR+/CD19+/CD13 wk+/CD33 wk+/CD56±
t(15;17)(q22;q11)	*PML*, *RARα*	AML-M3 (rare M1 or M2)	CD45+/CD34–/HLA-DR–/CD19–/CD2±/CD13+/CD33+
t(11;17)(q23:q11)	*PLZF*, *RARα*	AML-M3-like	CD45+/CD34–/HLA-DR–/CD19–/CD2±/CD13+/CD33+
inv(16)(p13q22)	*MYH11*, *CBFβ*	AML-M4Eo (some M2)	CD45+/CD34+/HLA-DR+/CD19–/CD2+/CD13+/CD33+/CD14±

Abbreviations: cy, cytoplasmic; s, surface; wk, weak.

change recognized by the MIC classification. The MIC Cooperative Group did not test their classification before its publication. Instead, they recommended that cooperative groups investigate the relationship of specific chromosomal abnormalities to laboratory features and treatment response. It would take another 15 yr before the next morphologic, immunologic, and cytogenetic classification of acute leukemias would be proposed. Despite the insight it provided into the potential clinical significance of chromosomal abnormalities in AML, the MIC classification was not incorporated into cooperative group studies of acute leukemia in the United States.

5. IMMUNOLOGIC INVESTIGATIONS AND CLASSIFICATION OF ACUTE LEUKEMIA

5.1. Lymphoblastic Leukemia

Immunologic studies or immunophenotyping of acute leukemia serve several purposes. Primary among these is to establish or confirm the lineage of a leukemic process. Multiparameter flow cytometric immunophenotyping is also useful for distinguishing acute leukemia from benign proliferations, such as virus-associated lymphoid proliferation or lymphoid regenerative processes following chemotherapy. The immunologic features of a leukemic process may provide prognostic information. As discussed below, expression of CALLA (or CD10) by T-ALL is associated with an improved clinical outcome. Lastly, immunophenotyping is a quick and sensitive technique for detecting small numbers of leukemic blasts in extramedullary sites or in the marrow and blood following treatment (minimal residual disease).

The first immunologic classifications of acute leukemia separated lymphoblastic from myeloblastic lineages and recognized B- and T-lineage ALL subtypes. The first indication that the stage of leukemic cell differentiation might have prognostic significance came from studies of pediatric B-lineage ALL *(14,16–18).* Subsequent immunologic classifications of ALL followed the development of monoclonal antibodies to cell lineage-associated and differentiation antigens. The production of clinically friendly flow cytometers with multiparameter analysis software complemented the availability of leukocyte monoclonal antibodies. With these new leukocyte reagents and flow cytometers, stages of leukocyte differentiation were delineated in ways not possible with the light microscope. These advances were used to develop new and more useful classifications of leukemias. Indeed, contemporary classifications of ALL correspond to normal stages of B- and T-cell maturation (Table 2) *(21).*

Early clinical investigations suggested that the stage of leukemic cell differentiation correlated with response to treatment. For example, early studies of childhood B-lineage ALL showed a poorer treatment outcome for pre-B-ALL compared with early pre-B-ALL (22). Subsequent combined immunophenotype and cytogenetic findings showed that this difference in outcome was due to a chromosomal t(1;19)(q23;p13) translocation that is exclusively associated with pre-B-ALL (23,24). More intensive therapy of pre-B-ALL with the t(1;19) translocation now results in treatment outcomes approaching that of early pre-B-ALL. In another example, expression of CALLA (or CD10) was associated with good responses to treatment. However, subsequent cytogenetic findings and improved chemotherapy treatments mitigated the independent prognostic importance of CD10 expression in B-lineage ALL. Clinical studies show that the leukemic cells of most patients with CD10-negative B-lineage ALL have a rearrangement of the *MLL* gene due in some cases to a t(4;11)(p22;q23) translocation (Table 3), a frequent chromosomal abnormality of ALL in patients younger than 12 mo of age. Subsequent studies revealed that chromosome 11q23 translocations, in particular t(4;11), are strong predictors of a poor treatment response that override the predictive importance of CD10 expression (25). Other reports suggest that the intensity of CD45 expression is correlated with a leukemic cell hyperdiploid karyotype (26,27). Associations of leukemic blast expression of other antigens with clinical behavior have not been confirmed by rigorous studies that carefully evaluated the influence of cytogenetic or molecular genetic abnormalities. Immunophenotyping studies have revealed characteristic antigen expression profiles that point to chromosomal abnormalities with prognostic significance but not with the accuracy of cytogenetic or molecular techniques (Table 3). In general, chromosomal abnormalities have largely nullified the usefulness of dividing B-lineage ALL into subgroups based on immunophenotype.

The value of recognizing subtypes of T-ALL by immunophenotyping is more controversial. Similar to B-lineage ALL, T-ALL has been divided into subgroups corresponding to phases of normal T-cell maturation (Table 2) *(21)*. However, attempts to identify immunophenotypic subtypes of T-ALL with prognostic significance have been largely unsuccessful. Previous studies in which T-ALL was classified as early (CD7+,

cytoplasmic CD3+, surface CD3–, CD4–, CD8–, and CD1–), mid or common (CD7+, cytoplasmic CD3+, surface CD3– or weak, CD4+, CD8+, CD1+), or late (surface CD3+, CD1–, CD4+ or CD8+) found that up to 25% of T-ALL cases have antigenic profiles that do not easily fit into a thymic stage of maturation. Furthermore, classifications based on normal T-cell differentiation are largely unsuccessful for predicting response to treatment. Similarly, the prognostic significance of individual antigen expressions by T-ALL blasts, such as CD3, CD2, CD5, and CD34, varies among several large clinical studies *(28–39)*. The disparities may be caused by differences in immunophenotyping methodologies and interpretations or differences in treatment. Multivariant analyses of patients with T-ALL at St. Jude Children's Research Hospital and the Pediatric Oncology Group concur that older age and lack of CD10 expression are independently associated with a poor clinical outcome *(28–30)*. In contrast to B-lineage ALL, characteristic antigen expression profiles in T-ALL are not associated with chromosomal abnormalities *(28)*. As discussed later, gene-expression profiling may point to unique antigenic expressions resulting from genetic abnormalities of leukemic T cells.

5.2. Acute Myeloid Leukemia

Immunophenotyping studies of AML are hampered by the relative lack of monoclonal antibodies to lineage-specific antigens. Additionally, antigen expression profiles of AML only partially correlate with stages of normal marrow myeloid, monocytic, or megakaryocytic differentiation *(40–43)*. The relatively poor correlation is largely owing to asynchronous antigen expression or differences in antigen intensity (intralineage infidelity) with leukemic cell differentiation. Similar to lymphoblastic leukemias, aberrant lymphoid-associated antigen expression (interlineage infidelity) is relatively common and often characteristic of certain cytogenetic abnormalities (Table 3). Older studies based on single-parameter immunophenotyping were inadequate for matching leukemic cell antigen expression with FAB AML subgroups *(44,45)*. However, multiparameter flow cytometric analysis may be more accurate than classic morphologic and cytochemical studies in identifying the lineage(s) involved in a case of AML *(46–52)*. With this approach, leukemic cells can be discriminated from normal hematopoietic cells. Light scatter and CD45 intensity expression can be combined to recognize characteristic patterns that correspond to the FAB AML subtypes. For most practical purposes the primary value of immunophenotyping in AML is to identify megakaryoblastic leukemia and AML subtypes that do not produce enzymatically active myeloperoxidase (AML M0). Although several large studies of adult and pediatric AML do not show any predictive value of the expression of individual leukocyte antigens, this issue continues to be debated *(52–54)*. Expression of CD7, high levels of CD34, or multidrug-resistant antigens, such as p180, may correlate with poor clinical outcomes in adult patients with AML, but such observations have not been used in planning patient treatment *(55–59)*.

5.3. Acute Leukemia with Aberrant Antigen Expression

Current evidence strongly supports the concept that leukemia represents the clonal expansion of a single transformed cell and that most leukemic processes mirror stages of normal leukocyte differentiation. Nonetheless, previous immunologic and molecular studies show that some acute leukemias can display features of one or more hematopoietic lineages (lineage infidelity). Acute leukemias whose blasts simultaneously show characteristics of more than one lineage (e.g., lymphoid plus myeloid) have been termed *acute mixed lineage*, *hybrid, chimeric*, or *biphenotypic leukemia (60–65)*. These leukemias should not be confused with the rare cases comprising two or more phenotypic but not necessarily genotypic lineages, variously termed *biclonal*, *bilineal*, or *oligoclonal leukemia*. The leukemias with mixed lineage, hybrid, or biphenotypic features can be defined by morphologic, cytochemical, ultrastructural, and molecular studies, but in most instances they are identified by immunologic studies.

Investigations of the past decade support the concept of two broad categories of acute leukemias with disparate expressions of lineage-associated features. Acute leukemias in the most common category have distinct immunologic, genotypic, and clinical features characteristic of a strong commitment to a single lineage but with one or several aberrant features of another lineage. These include ALL-expressing myeloid-associated antigens (My^+ALL) and AML with lymphoid-associated antigen expression (Ly^+AML). The second category of leukemias displays a mixture of genotypic and antigenic features that make it unclear whether the leukemic blasts are committed to a single lineage of differentiation (i.e., true mixed, hybrid, or biphenotypic leukemias). Recognition of these two categories is clearly a useful advance in leukemia classification, but confusion remains as to their diagnostic criteria, nomenclature, optimal treatment, and prognostic significance. This lack of agreement can be attributed to inconsistencies among studies of these unusual cases, including the patient population studied (pediatric, adult, or a mixture of both), different laboratory methodologies, stringency of the immunologic criteria for defining commitment to lymphoid or myeloid differentiation, and treatment approaches *(64,65)*. Chief among these appears to be the immunologic criteria for defining commitment to the lymphoid or myeloid lineage. For example, definitions vary depending on the immunologic methods employed: single or multiparameter flow cytometry; fluorescence microscopy or immunohistochemistry; the number and type of monoclonal antibodies used; inclusion of antigens that are not lineage-restricted [e.g., CD4, CD11b, CD15, CD10, or terminal deoxynucleotidyl transferase (TdT)]; source and condition of the leukemic samples (e.g., marrow or blood; fresh, old or cryopreserved cells); and the criteria for positive or negative antigen expression.

The criteria used at St. Jude Children's Research Hospital to define My^+ALL, Ly^+AML, and "true mixed" or biphenotypic leukemia are presented in Table 4. The central feature of this classification is the identification of antigens that substantiate lymphoid and myeloid lineage commitment. As shown in Fig. 1, B-lineage ALL is diagnosed when leukemic blasts express CD19 plus CD22 and cytoplasmic CD79α or immunoglobulin, and no cytoplasmic CD3 or myeloperoxidase. The leukemic cells of T-ALL express CD7 plus either surface or cytoplasmic CD3 but do not coexpress surface CD19 and CD22 or cytoplasmic CD79α and myeloperoxi-

Table 4
SJCRH Criteria for My+ ALL, Ly+ AML, and Biphenotypic Leukemia

Ly+ AML[a]

1. Leukemic blasts are MPO+[b] (or ANB+ if AML M5)
2. Leukemic blasts are cyCD3–
3. Leukemic blasts are cyIgμ– and do not coexpress CD22 plus cyCD79α–
4. Leukemic blasts express ≥1 lymphoid-associated antigens: CD2, CD5, CD7, CD19, CD22, CD56, cyCD79α

Biphenotypic acute leukemia

Myeloid/B-lineage biphenotypic acute leukemia:
Leukemic blasts coexpress MPO[b] and cyIgμ, or MPO[b] and cyCD79α plus CD22
Myeloid/T-lineage biphenotypic acute leukemia:
Leukemic blasts coexpress MPO[b] plus cyCD3
Mixed B- and T-lineage acute leukemia:
Leukemic blasts coexpress cyCD3 plus cyIgμ, or cyCD3 and cyCD79α plus CD22

B-lineage My+ALL[a]

1. Leukemic blasts are CD19+ plus CD22+ or cyCD79α+ or cyIg μ+
2. Leukemic blasts are cyCD3–
3. Leukemic blasts are MPO–[b]
4. Leukemic blasts express ≥1 myeloid-associated antigens: CD13, CD14, CD15, CD33, CD36, or CD65

T-lineage My+ ALL[a]

1. Leukemic blasts are CD7+ and cyCD3+
2. Leukemic blasts are CD22–
3. Leukemic blasts are MPO–[b]
4. Leukemic blasts express ≥1 myeloid-associated antigens: CD13, CD14, CD15, CD33, CD36, CD65, CD79α[wk]

Abbreviations: SJCRH, St. Jude Children's Research Hospital; Ly+ AML, acute myeloid leukemia expressing lymphoid (Ly)-associated antigens; My+ ALL, acute lymphoid leukemia expressing myeloid (My)-associated antigens; MPO, myeloperoxidase; ANB, α-naphthyl butyrate esterase; cyCD, cytoplasmic antigen expression; wk, weak.
[a]All four criteria must be fulfilled.
[b]Confirmed by cytochemical, anti-MPO, or ultrastructural study.

dase. AML is diagnosed when leukemic blasts express myeloperoxidase or in its absence, two or more myeloid-associated antigens, including CD13, CD15, CD33, or CD65 but not cytoplasmic CD3, immunoglobulin, or simultaneously CD19, CD22, and cytoplasmic CD79α. A case of My+ ALL would have the antigenic expression profile defined for B- or T-lineage ALL plus one or more myeloid-associated antigens, such as CD13, CD15, CD33, and CD65 but not myeloperoxidase. A case of Ly+ AML will display the antigen profile described above for AML plus one or more lymphoid-associated antigens but not cytoplasmic CD3 or coexpression of surface CD19, CD22, and cytoplasmic CD79α.

Several large studies of childhood My+ ALL show that myeloid-associated antigen expression does not have independent prognostic significance *(66–71)*. Other studies have failed to consider the impact of genetic abnormalities on clinical outcome in My+ cases. For example, atypical expression of the myeloid-associated antigen CD15 is common in B-lineage ALL with t(4;11), a translocation that confers a poor outcome in infants and older children independently of immunophenotype *(72)*. By contrast, patients with B-lineage ALL with t(12;21)(p12;q21) have a favorable outcome regardless of the presence or absence of the myeloid-associated antigens CD13 or CD33. The clinical importance of My+ ALL in adults is still unknown *(75–78)*.

Most studies of pediatric and adult Ly+ AML find no significant effect of lymphoid antigen expression on clinical outcome except for CD7-positive AML *(65,69,79–82)*. Similar to B-lineage ALL, the aberrant lymphoid antigen expression is largely associated with certain chromosomal abnormalities. For example, favorable cases of AML with t(8;21)(q22;q22) and inv(16)(p13q22) almost always express the lymphoid-associated antigens CD19 and CD2, respectively whereas CD7 is associated with MDS-related and secondary AMLs that frequently display abnormalities of chromosome 7 *(83–89)*.

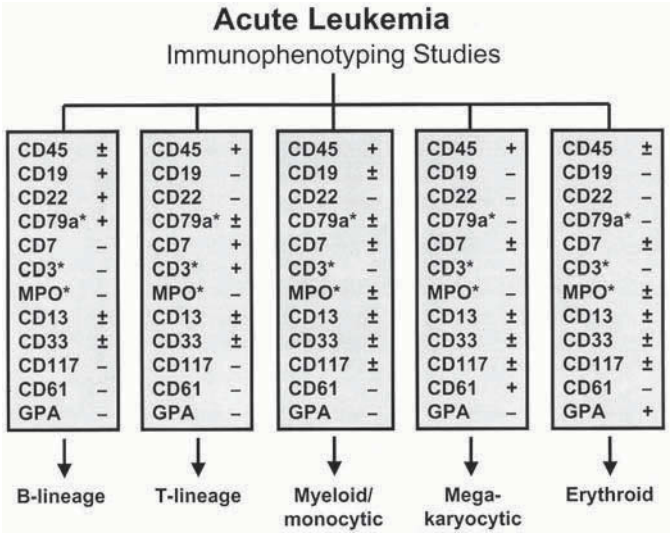

Fig. 1. Basic screening panel for immunophenotyping the major lineages of acute leukemia. The asterisks indicate cytoplasmic antigen expression. Over 98% of B-lineage acute lymphoblastic leukemia (ALL) cases will coexpress CD19, CD22, and CD79α, whereas T-lineage ALL and acute myeloid leukemias (AML) may express CD19 or rarely CD19 plus CD79α, but not CD19 plus CD22 plus CD79α.

An immunophenotypic diagnosis of true mixed or preferably biphenotypic leukemia is considered when the leukemic blasts express MPO plus CD3, MPO plus immunoglobulin, or MPO plus surface CD19, CD22, and cytoplasmic CD79α (Table 4). The European Group for the Immunological Characterization of Leukemia (EGIL) proposed a scoring system for defining biphenotypic leukemias *(90)* in which points are assigned to a lymphoid or myeloid antigen based on its degree of lineage specificity (Table 5). Biphenotypic leukemia is diagnosed when scores exceed 2 for the myeloid lineage plus 2 for

Table 5
EGIL Immunophenotyping Criteria
(Scoring System) for Biphenotypic Acute Leukemias

B-lineage	*T-lineage*	*Myeloid*	*Points*[a]
CD79α	cy/s CD3	MPO	2
CyIgμ	TCRα/β		
cy/sCD22	TCRγ/δ		
CD19	CD2	CD13	1
CD10	CD5	CD33	
CD20	CD8	CD65s	
	CD10	CD117	
TdT	TdT	CD14	0.5
CD24	CD7	CD15	
	CD1a	CD64	

Abbreviations: EGIL, European Group for the Immunological Characterization of Leukemia; cy, cytoplasmic; s, surface; TCR, T-cell receptor; MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase.

[a]Biphenotypic acute leukemia is defined by >2 points from the myeloid group and >2 points from the B-lineage or T-lineage group.

Data from ref. *90*.

either the B- or T-lineage. The preceding criteria defining biphenotypic leukemia are probably oversimplified, as evidenced by more sophisticated multiparameter flow cytometric analysis. For example, in our studies of such cases, two or more populations of leukemic blasts with discordant immunophenotype profiles may be present in a patient's leukemic specimen. Sometimes, only a minor number of leukemic blasts may have a biphenotypic immunotype, with the greater proportion of blasts demonstrating commitment to a single lineage. The clinical dilemma created by these observations is obvious. Thus, whereas pediatric and adult patients with biphenotypic leukemias appear to have a poor clinical outcome, it will be important to confirm this finding with standardized immunophenotyping methods and criteria for defining biphenotypic leukemia.

6. GENETIC CLASSIFICATION OF ACUTE LEUKEMIA

Studies of pediatric and adult leukemias have conclusively demonstrated the significant impact of genetic abnormalities on clinical response to treatment. Indeed, many specific chromosomal abnormalities have been described that frequently override the importance of morphologic, immunologic, and clinical features. Pediatric patients with ALL and underlying chromosomal hyperdiploidy >50 or t(12;21) have excellent treatment responses, whereas poorer clinical outcomes are associated with chromosomal hypodiplody <45, or t(1;19), t(9;22), or t(4;11) (Table 6). The t(12;22) and chromosomal hyperdiploidy >50 are uncommon in adults compared with children with ALL (Figs. 2 and 3) *(91,92)*, whereas t(9;22) is more common in adults (Fig. 3) *(92–94)*. In pediatric ALL, more intensive consolidation treatment is given to patients with poor-risk cytogenetic features. This risk-adapted therapeutic approach has been very successful and supports the inclusion of cytogenetics in any classification system for ALL *(8,95)*.

The strikingly different incidences of major cytogenetic abnormalities in adult vs pediatric ALL (Figs. 2 and 3) are not reiterated in AML (Fig. 4). Additionally, the clinical outcomes by cytogenetic group are similar for adult and pediatric patients with AML. Not surprisingly, characteristic morphologic and immunologic features are also associated with many of the nonrandom chromosomal abnormalities in AML (Table 3). Patients whose AML is defined by t(8;21), t(9;11), t(15;17), inv(16), or t(16;16) translocations fare significantly better than those with normal karyotypes, chromosomal 3q translocations or deletions, t(6;9), or monosomy 7 or 7q deletions. Indeed, the leukemic cell karyotype is the strongest prognostic factor in AML. Three cytogenetic risk groups—favorable, intermediate, and adverse—are widely accepted and currently considered in planning treatment *(96–101)*. However, different cooperative groups assign cytogenetic abnormalities to different prognostic subgroups (Table 7).

Despite the association of specific chromosomal abnormalities with clinical outcome, and the intriguing insights afforded by these defects, a clinically useful classification of acute leukemia based solely on cytogenetic studies is not practical for several reasons. The most obvious of these is that a significant number of ALL and AML cases do not have a chromosomal abnormality that defines a leukemic entity or predicts clinical outcome. In AML, most patients are in the intermediate-risk group (Table 7). Furthermore, it is highly probable that within well-defined cytogenetic risk groups, other (unrecognized) genetic lesions influence clinical outcome. For example, despite the relatively good response of myeloblastic leukemias with t(8;21) or inv(16), an unacceptable 40–50% of these patients are not cured with chemotherapy alone, for reasons other than the presence of known high-risk features. This strongly suggests an influence from additional genetic lesions in these leukemias. One possible cooperating genetic abnormality may be the *FLT3* internal tandem duplication (*FLT3* ITD). Recent investigations show that *FLT3* ITD is the most common genetic abnormality in AML, one that adds important prognostic information to all three genetic-risk groups *(102–108)*. The outcomes for patients with AML are significantly worse for those with *FLT3* IDT, but the significance of *FLT3* mutations appears to decline with age *(104–108)*. In one pediatric study, *FLT3* mutations were found in only the favorable and intermediate risk groups *(103)*. Although no study thus far has sufficient numbers of AML patients with favorable cytogenetic features, i.e., t(15;17), t(8;21), or inv(16), to say whether or not *FTL3* mutations are a confounding factor in predicting clinical outcome, it is possible that this or other genetic abnormalities influence treatment response. Thus, the present classification of three major cytogenetic AML risk groups may be an oversimplification and will be inadequate as a clinically useful classification of AML. Predictably, a more fully characterized genetic profile is required to build a useful genetics-based classification of acute leukemias (see the later discussion of gene expression profiling).

7. WHO CLASSIFICATION OF ACUTE LEUKEMIA

Investigations over the last 15 years have demonstrated the importance of immunologic and cytogenetic studies for classi-

Table 6
Cytogenetic Classification of Acute Lymphoblastic Leukemia (ALL)

Karyotype	*Genes involved*	*Leukemia subtype*	*Clinical prognosis*
Hyperdiploid >50[a]		Early pre-B- or pre-B-ALL	Favorable
t(12;21)(p12;q22)	*TEL*, *AML1*	Early pre-B- or pre-B-ALL	Favorable
t(1;19)(q23;p13)	*PBX1*, *E2A*	Pre-B-ALL	Good with intensified therapy
t(8;...)(q24;...)[b]	c-*MYC*,—[b]	Mature B-ALL (ALL-L3)	Favorable without central nervous system disease
t(11;19)(q23;p13.3)	*MLL*, *ENL*	Early pre-B- or T-ALL	Poor in patients <1 yr; favorable in T-ALL
t(4:11)(q21;q23)	*AF4*, *MLL*	Early pre-B-ALL	Poor in patients <1 or >10 yr of age
t(9;22)(q34;q11)	*ABL*, *BCR*	Early pre-B- or pre-B-ALL	Poor
Near haploid <30[a]		Early pre-B-ALL	Poor

[a]Chromosomes.
[b]Includes t(8;14)(q24;q32), t(2;8)(p12;q24), and t48;22)(24;q11) where heavy, κ, and λ immunoglobulin genes are involved on chromosomes 14, 2, and 22, respectively.

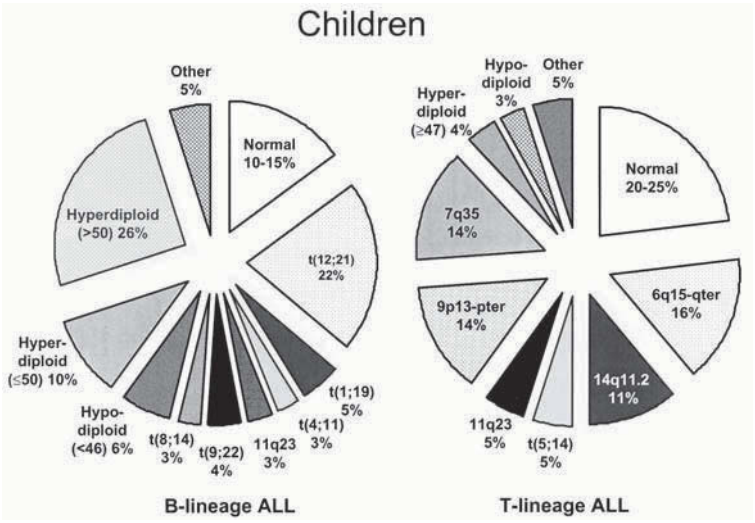

Fig. 2. Recurring chromosomal abnormalities in pediatric acute lymphoblastic leukemia (ALL) as detected by classic cytogenetics and fluorescense in situ hybridization.

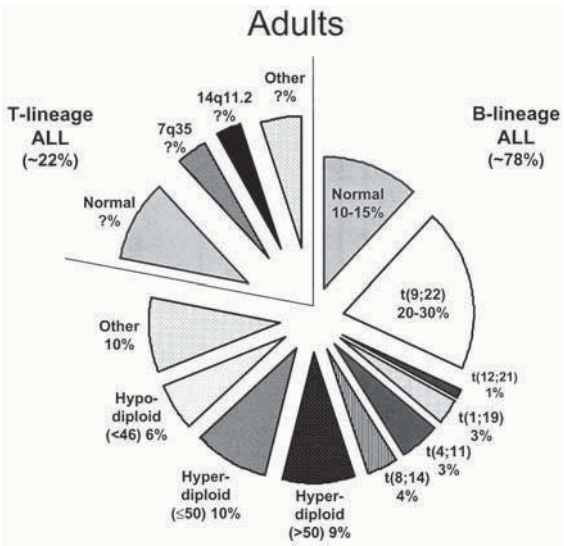

Fig. 3. Recurring chromosome abnormalities in adult acute lymphoblastic leukemia (ALL) as detected by classic cytogenetics and fluorescense in situ hybridization. The pie chart is divided into B- and T-lineage ALL with further subdivision into chromosomal abnormalities. Chromosome 7q abnormalities include translocations t(7;11)(q35;p13), t(7;10)(q35;q24), and others involving the *TCR*β gene. Chromosome 14q abnormalities include translocations t(11;14), t(10;14), t(8;14), and others involving the *TCR*α/*TCR*δ gene complex. The t(11;19) translocation involving *MLL* is the most common T-ALL abnormality of chromosome 11q23. Relative incidences in chromosome abnormalities for adult T-ALL all not available.

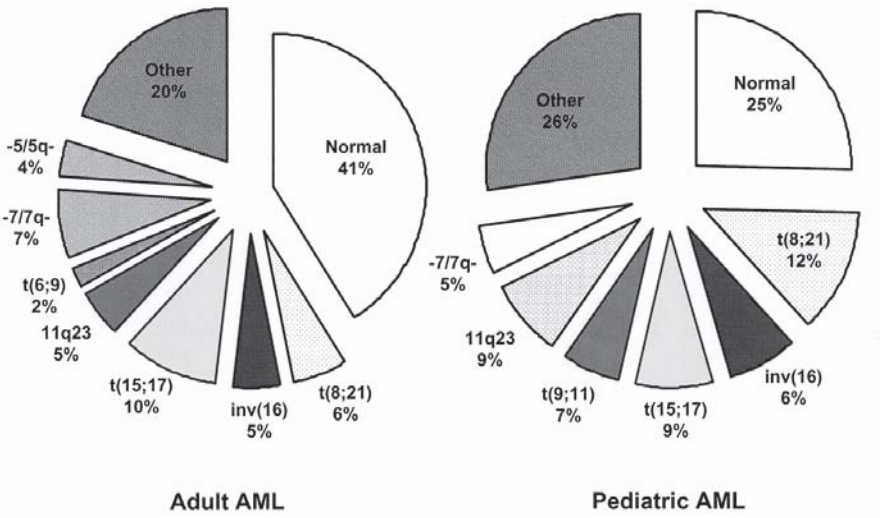

Fig. 4. Recurring chromosomal abnormalities in pediatric and adult acute myeloid leukemia (AML) as detected by classic cytogenetics and fluorescense *in situ* hybridization.

Table 7
Cytogenetic Classifications of Acute Myeloid Leukemia

Group	*CALGB (96)*	*MRC (97,98,100)*	*GAMLCG (101)*	*SWOG (99)*
Favorable	t(15;17)	t(15;17) with any abnormality	t(15;17)	t(15;17) with any abnormality
	inv(16)/t(16;16)/del(16)	inv(16)/t(16;16)/del(16q) with any other abnormality	inv(16)/t(16;16)	inv(16)/t(16;16)/del(16q) with any other abnormality
	t(8;21)	t(8;21) with any other abnormality	t(8;21)	t(8;21) without del(9q) or complex karyotype
Intermediate	Normal karyotype	Normal karyotype	Normal karyotype	Normal karyotype
		+8, –Y, +6, der(12p)	Other abnormalities	+8, –Y, +6, der(12p)
		11q23 abnormality		
		del(9q) or del(7q) without other abnormality		
		Complex karyotypes (≥3 but <5 abnormalities)		
		All abnormalities of unknown prognostic significance		
Unfavorable	Other abnormalities	–5/del(5q) –7	–5/del(5q). –7/del(7q)	–5/del(5q). –7/del(7q)
		inv(3q), del(9q), 17p abnormality	inv(3), 17p	inv(3), 17p abn, 20q, +13,
		t(6;9)	12p	t(6;9)
		t(9;22)		t(9;22)
			11q23	11q23 abnormality
				(8;21) with del(9q) or complex karyotype
		Complex karyotypes with ≥5 abnormalities	Complex karyotype	Complex karyotypes with ≥3 abnormalities
Unknown	—	—	—	All other clonal karyotypes with <3 chromosomal abnormalities

Abbreviations: CALGB, Cancer and Leukemia Group B; MRC, Medical research Council; SWOG, Southwestern Oncology Group; GAMLCG, German AML Cooperative Group.

Table 8
World Health Organization (WHO) Classification of Acute Leukemia with Corresponding FAB Classification Subtypes

WHO classification[a]	*Corresponding FAB subtypes*[b]
Precursor lymphoblastic leukemia/lymphoblastic lymphoma	
Precursor B-cell acute lymphoblastic leukemia/lymphoma	L1, L2
Precursor T-lymphoblastic leukemia/lymphoblastic lymphoma	L1, L2
Burkitt's lymphoma/leukemia	
Endemic Burkitt's lymphoma/leukemia	L3
Sporadic Burkitt's lymphoma/leukemia	L3
Immunodeficiency-associated Burkitt's lymphoma/leukemia	L3
AML with recurrent genetic abnormalities	
AML with t(8;21)(q22;q22); *AML1-ETO*	M2>M1>M4>M0
AML wth abnormal marrow eosinophilia and inv(16)(p13q22) or t(16;16)(p13;q22): *CBFβ-MYH11*	M4Eo>M4>M2>M1
Acute promyelocytic leukemia with t(15;17)(q22;q12); *PML-RARα*	M3>M2>M1
AML with 11q23 abnormalities; *MLL* rearrangements	M5>M4>M2>M1>M0
AML with multilineage dysplasia	
Following a myelodysplastic syndrome or myeloproliferative disorder or without antecedent myelodysplastic syndrome	M2>M4>M6
AML and myelodysplastic syndrome, therapy-related	
Alkylating agent-related	M2>M4>M6
Topoisomerase type II inhibitor-related	M5>M4>M2>M1
Other types	
AML not otherwise categorized	
Acute myeloid leukemia minimally differentiated	M0
Acute myeloid leukemia without maturation	M1
Acute myeloid leukemia with maturation	M2
Acute myelomonocytic leukemia	M3
Acute monoblastic leukemia	M4
Acute erythroid leukemia	M5
Acute megakaryoblastic leukemia	M7
Acute basophilic leukemia	—
Acute panmyelosis with myelofibrosis	M7; ? M1; ? MDS
Myeloid sarcoma	—

Abbreviations: FAB, French–American–British;MDS, myelodysplastic syndrome.
[a]For details, see ref *6*.
[b]For details, see refs. *3* and *4*.

fication and treatment of pediatric lymphoblastic malignancies. The value of these studies for the classification of adult and pediatric acute myeloid malignancies has come to light more recently. The indispensability of these studies has not been lost on the framers of the recently introduced WHO Classification of Tumors of Hematopoietic and Lymphoid Tissue *(6)*. Whereas the FAB classification attempted and partly achieved a standardized morphologic classification of hematopoietic malignancies, and the MIC classification recognized the importance of several major nonrandom cytogenetic abnormalities, the newer WHO classification purports to go beyond these classifications by continuously recognizing new clinically relevant molecular genetic lesions. Interestingly, the newest WHO classification system continues to rely heavily on classic morphology for identification of a substantial proportion of AML cases, since understanding of the genetic basis of leukemogenesis is largely incomplete. Indeed, the morphologic features of leukemic blasts are but one manifestation of their underlying genetic abnormalities.

The WHO classification stratifies hematopoietic neoplasias by major lineage: lymphoid, myeloid, histiocytic/dendritic, and mast cell. Within each major group, distinct disease entities are defined by a combination of clinical syndrome and morphologic, immunophenotypic, and genetic features (Table 8) *(9)*. A cell of origin is suggested for each of the acute leukemias. As admitted by the authors of the WHO classification, this cell often represents the stage of differentiation of the malignant cells rather than the cell in which the initial transforming event occurs. In some myeloid leukemias, the cell of origin is known to be a multipotential stem cell, even though most of leukemic cells may be committed to a particular lineage or a late stage of myeloid differentiation (e.g., AMLs arising from MDS).

The WHO classification departs from the FAB and MIC classifications by combining ALL with lymphoblastic lymphomas. The authors conclude that laboratory evidence justifies the concept that B-precursor and T-lineage ALL and B- and T-lymphoblastic lymphomas, respectively, as well as ALL-L3 and Burkitt's lymphoma, are different clinical manifestations

of the same neoplasm *(9)*. Surprisingly, this line of reasoning is not applied to myeloid sarcomas, which are recognized as an entity related to but separate from other AMLs. Major differences between the WHO and FAB classifications of acute leukemias and myelodysplastic syndromes include:

- Replacing the morphologic terms of L1 and L2 ALL with an immunologic classification consisting of precursor-B and precursor-T lymphoblastic leukemias that are further subgrouped by cytogenetic abnormalities
- Grouping L3 ALL with Burkitt's lymphoma
- Lowering the blast count from 30 to 20% for the diagnosis of AML, with elimination of the myelodysplastic subgroup of refractory anemia with excess blasts in transformation (RAEB-IT)
- Revision of the MDS subdivision based on number of dysplastic lineages, presence of ringed sideroblasts, and blast percentage
- Recognition of distinct cytogenetic AML subtypes
- New category of AML with multilineage dysplasia with or without an antecedent MDS
- New category of Therapy-Related AML
- New category of Acute Leukemia of Ambiguous Lineage
- Inclusion of a pure erythroid leukemia (M6b) in the AML Not Otherwise Categorized subgroup
- Recognition of the rare acute basophilic leukemia also in the AML Not Otherwise Categorized subgroup.

The authors of the WHO classification invested considerable time in its development, cautiously incorporating current biologic insights and discarding irrelevant or outdated information. Although the proposed WHO classification is an improvement over previous classifications, critical questions remain as to its laboratory application and clinical usefulness. Potential problems revolve around the standardization of morphologic criteria. Lowering the blast count from 30 to 20% for the diagnosis of AML will not solve the dilemma of distinguishing *de novo* AML from MDS or the difficulty that morphologists often experience in differentiating leukemic blasts from slightly more mature cells (e.g., myeloblasts from early promyelocytes). Elaborate previous proposals for distinguishing among type I, II, and even III blasts have not been useful *(7)*. Thus, the problem that existed with the FAB requirement of 30% blasts for defining AML will persist. A similar problem exists in defining the morphologic criteria for dysplasia. Not infrequently, dysplastic changes may be subtle or present in only a small percentage of cells, undoubtedly leading to problems in differentiating AML with Multilineage Dysplasia from AML Not Otherwise Categorized. The WHO classification attempts to clarify the difference between these two categories by requiring that the latter show dysplasia in at least 50% of cells. However, a case with <50% dysplastic cells, 40% for example, will be excluded from the AML with Dysplasia Category. Supporting data for such separation do not exist. If multilineage dysplasia in AML is truly a unique feature, why artificially separate AML with less or more than 50% dysplastic cells? Furthermore, it should be remembered that investigators disagree over the clinical significance of AML presenting with multilineage dysplasia.

Another problem facing investigators who plan to use the WHO classification will be the standardization of immunologic and genetic testing and the criteria for interpreting these tests. How will new discoveries of genetic abnormalities be incorporated into the WHO classification in a timely fashion? At this writing there is already evidence that point mutations (e.g., *PT53*, *FLT3*, and *P16*), predict a poorer therapeutic response. Quite likely, some cooperative groups but not others will base their treatment programs on such discoveries, making intergroup comparisons difficult. Finally, the WHO classification was not subjected to clinical testing before being introduced to the international community of hematologists and oncologists. Hence, its reproducibility and the methods best suited to acquiring informative results will not be clear for several more years. Even with these caveats, the WHO classification of acute leukemias should improve comparisons among different study groups.

8. GENE EXPRESSION PROFILING IN THE CLASSIFICATION OF ACUTE LEUKEMIA

The phenotypic and cytogenetic diversity of acute leukemia is accompanied by a corresponding diversity in gene expression patterns. Gene expression profiling using cDNA microarrays permits simultaneous analysis of multiple gene markers and has been used successfully to categorize a variety of malignancies *(109–115)*. Advances in bioinformatics are not only making it possible to categorize leukemias into recognizable morphologic and cytogenetic subtypes but also show strong promise of being able to recognize additional types that may aid in predicting disease course *(110,116)*. The ability of gene profiling to identify currently recognized subtypes of leukemia is not unexpected, since gene expressions dictate morphologic, immunophenotype, and other leukemic cell manifestations of ALL and AML. Ultimately, this approach to leukemia classification may allow disease aggressiveness and treatment responsiveness to be reliably predicted for individual cases.

The first report of gene profiling by DNA microarray analysis, specifically applied to human leukemia, demonstrates the ability of this technology to distinguish AML accurately from ALL, as well as B- from T-lineage ALL *(110)*. In this study, investigators were able to identify 50 genes that would serve as a class predictor of AML or ALL in the vast majority of cases, with 100% accuracy. In a more recent study of a large number of ALL samples, investigators accurately distinguished B-lineage from T-lineage ALL, while identifying several important prognostic cytogenetic subgroups of B-lineage ALL—hyperdiploid >50, t(12;21), t(1;19), and *MLL* rearranged—with 95–100% accuracy *(116)*. A novel group of B-lineage ALL cases with a unique gene profile were also identified. Surprisingly, initial analysis of the study data strongly suggested that gene profiling is capable of predicting those patients with ALL who will fail contemporary multiagent chemotherapy. Gene profiling also appears to be a promising technique for predicting resistance to the tryosine kinase-inhibiting agent ST1571. In a study of ALL with t(9;22) translocations, the gene expression profiles discriminated all patients who were sensitive to ST1571 from those resistant to this kinase inhibitor *(117)*. In another study of pediatric T-ALL patients, gene expression signatures delineated novel molecular pathways that may drive the malignant transformation of developing T cells *(118)*. Using oligo-

nucleotide microarrays, these investigators identified several gene expression signatures indicative of leukemic cell arrest that corresponded to specific stages of normal thymocyte development: *LYL1*$^+$, *HOX11*$^+$, and *TAL1*$^+$ signatures, corresponding to pro-T, early cortical, and late cortical thymocyte stages, respectively. Activation of *HOX11L2* was further identified as a novel event in T-ALL leukemogenesis. *HOX11* expression was associated with a favorable prognosis, whereas activation of *TAL1*, *LYL1*, and *HOX11L2* was found to predict a poorer treatment outcome.

In yet another study, gene expression profiles showed that ALL cases possessing a rearranged *MLL* gene have a highly uniform and distinct gene expression pattern that distinguishes them from conventional ALL or AML *(119)*. The leukemias with rearranged *MLL* genes expressed some lymphoid- and myeloid-specific genes, but at lower levels than other cases of ALL and AML. These leukemias also expressed genes characteristic of progenitor cells. The investigators contend that their observations support the derivation of *MLL*$^+$ leukemia from a very early B-cell progenitor that has the potential to differentiate in either the lymphoid or myeloid/monocytic pathway. This study also supports a model of leukemogenesis in which a specific chromosomal translocation results in a distinct type of leukemia, rather than a model in which all cells bearing translocations converge on a common pathway of leukemogenesis.

Gene-expression profiling will no doubt lead to other remarkable discoveries in acute leukemia. For example, this molecular genetic strategy will make it possible to examine the full spectrum of deletions and additions of genetic loci, mutations, and rearrangements in tyrosine kinases, hematopoietic transcription factors, and even single nucleotide polymorphisms—all of which can influence response to treatment. Thus, with gene profiling, one can produce a fingerprint for each leukemia patient that will direct optimal therapy and predict clinical outcome. Leukemia gene-expression fingerprints may in fact replace classifications of acute leukemia as we now know them. The present limitations of microarray technology include its cost and availability. Most reports of gene profiling in acute leukemia are retrospective, with unblinded analyses, and focus on samples with a high percentage of leukemic blasts. Whether the spectacular results of these initial reports can be reproduced prospectively and performed on the entire spectrum of leukemic samples, including those with low blast cell percentages, remains to be seen.

9. SUMMARY AND RECOMMENDATIONS

A classification of acute leukemia should be reproducible, should impart an understanding of leukemogenesis and clinical behavior, and should be clinically relevant. Each of the classifications presented above fails to satisfy all three of these requirements fully. The WHO classification is a theoretical improvement over all the others, but its reproducibility and clinical relevance have not been tested. It is not even clear that any single classification would satisfy all users. The WHO classification attempts to categorize acute leukemias by combining clinical and biologic features. As a result, its biologic criteria are oversimplified and may not be relevant as new therapies are developed. It may be more useful to devise separate clinical and biologic classifications. For example, the laboratory investigator would be most interested in a detailed biologic classification, whereas the physician would favor a more clinically relevant categorization. Indeed, with some recent exceptions, acute leukemia treatments are not so refined as to require a classification that would accommodate every conceivable subtype of ALL or AML.

Presently, the WHO classification offers the best system for comparing clinical trials. However, to be more relevant, it must be modified to include additional chromosomal or molecular genetic abnormalities that are clinically relevant [e.g., t(11;17) and t(8;16) in AML]. The Multilineage Dysplasia category of AML will be difficult to reproduce among different investigators and needs further refinement. The AML Not Otherwise Categorized subgroup is a waste bin of different leukemias and will no doubt vary in size and complexity depending on the skill of the morphologist and the availability of sophisticated molecular assays.

It may well be that the explosion of new information coming from gene expression profiling studies will render the WHO classification obsolete before it can be fully tested in clinical trials. This new technology will undoubtedly provide a more exact model of leukemogenesis, which in turn may suggest new modes of treatment requiring revised classifications of the lymphoid and myeloid leukemias. We can look forward to the day when each patient's leukemia will be classified by its gene expression profile. Treatment will be based not only on this profile, but also on the patient's intrinsic genetic profile, which largely determines how he or she will respond to therapy.

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CHEMOTHERAPEUTIC STRATEGIES

II

INFANT LEUKEMIAS

A

3 Biology and Treatment of Infant Leukemias

Perspective 1

ROB PIETERS

CONTENTS

1. INTRODUCTION

Although an age limit of 18 or even 24 mo is used in some studies, infant leukemia is usually defined as leukemia occurring in the first year of life. Infant acute leukemia differs from acute leukemias in older children and adults in many ways, for example, in the distribution of lymphoid and myeloid leukemias. Also, many risk factors are more prevalent in infants, including certain clinical characteristics as well as immunophenotypic, cytogenetic, and molecular genetic features. The important consequences of these differences for prognosis and treatment justify a separate chapter for acute leukemia in the youngest age category.

2. EPIDEMIOLOGY

After neuroblastoma, leukemias are the most common malignancy in infants. Data from the U.S. Surveillance, Epidemiology, and End Results (SEER) program of the USA suggest that the incidence of both infant acute lymphoblastic leukemia (ALL) and infant acute nonlymphoblastic leukemia (ANLL) has increased slightly over the last few decades *(1)*. Although this increase may be real, changes in case reporting practices may be a contributing factor. Infants account for about 4% of childhood ALL *(2–7)* and about 10% of childhood ANLL cases *(5,8–11)*. Whereas ALL has a higher incidence than ANLL in older children, the incidence of ALL and ANLL in infants is about equal. In contrast to the predominance of male sex in older children with ALL, there is a slight predominance of girls in infant ALL *(12)*. In studies of nonselected series of infants with ALL, female patients have outnumbered male patients *(4,5,7,13–18)*. Two hundred fifty-five of 566 infant ALL cases (45%) were diagnosed in the first half-year after birth *(4,13–15,17,18)* (Table 1).

3. ETIOLOGY

3.1. Prenatal Leukemogenesis

At least two independent mutations are thought to be necessary to cause leukemia. The first is thought to take place *in utero* for most of childhood leukemia cases in general *(19,20)*. Postnatal events are required for the full development of leukemic phenotype. However, in infant leukemia, all necessary genetic events may have occurred *in utero*. Indirect evidence for this hypothesis includes the sometimes very early onset of infant leukemia [e.g., fetal death owing to *MLL* gene rearrangement in AML *(21)*] and the very high rate of concordance of infant leukemia in identical monozygotic twins if one of the children developed leukemia in the first year of life *(19)*. Ford et al. *(22)* found in three sets of infant twins with leukemia that all twin pairs shared identical *MLL* gene rearrangements in their malignant cells, with different additional gene abnormalities across the three pairs of twins. These leukemias presumably spread from one twin, in whom the

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Table 1
Clinical Characteristics of Infant ALL at Diagnosis

Characteristic	*No. of patients*[a]		*Reference*
Gender (Male/female, with percentage of female cases)	17:12	(41)	*7*
	51:55	(52)	*4*
	15:14	(48)	*5*
	106:128	(55)	*13*
	31:51	(62)	*14*
	21:12	(36)	*15*
	13:15	(54)	*16*
	38:50	(57)	*17*
	14:9	(39)	*18*
Age (≤6 mo/> 6 mo, with percentage ≤ 6 mo)	34:48	(41)	*14*
	105:129	(45)	*13*
	20:13	(61)	*15*
	50:56	(47)	*4*
	38:50	(43)	*17*
	8:15	(35)	*18*
	13:7	(65)	*7*
	15:13	(54)	*16*
	48:34	(58)	*46*
Leukocyte count ($\times 10^9$/L) (<50/>50 and <100/>100, with percentage of cases >50 and >100)	35:46	(57) and	
	49:32	(39)	*14*
	11:22	(67) and	
	15:18	(55)	*15*
	84:150	(64) and	
	?	—	*13*
	5:18	(78) and	
	10:13	(57)	*18*
	10:19	(66) and	
	?	—	*7*
	?	— and	
	46:50	(52)	*4*
	29:59	(67) and	
	43:45	(51)	*17*
	36:46	(56) and	
	48:34	(41)	*46*
CNS involvement (no/yes, with percentage of positive cases)	61:21	(26)	14
	29:4	(12)	15
	207:26	(11)	13
	20:3	(13)	18
	12:12	(50)	7
	81:25	(24)	4
	78:9	(10)	17

[a]Percents are in parentheses.

disease originated, to the other by the shared circulation in the monochorionic placenta. Direct evidence for the prenatal origin of infant leukemia was demonstrated by Gale et al. *(20)*, who detected unique MLL-*AF4* fusion sequences in the neonatal blood spots of Guthrie cards from infants who developed ALL between 5 mo and 2 yr.

3.2. Topoisomerase Inhibitors

MLL gene rearrangements are found not only in infant leukemia, but also in secondary myeloid leukemia associated with prior exposure to epipodophyllotoxins *(23)*, among other inhibitors of topoisomerase II. Thus, *in utero* exposure of the fetus to topoisomerase II inhibitors may play a critical role in the development of infant leukemia *(24)*. Potential topoiomerase II DNA binding sites were found near the breakpoints of both chromosomes involved in a t(9;11) positive infant ANLL case *(25)*. A large number of natural and synthetic topoisomerase II inhibitors exist, including flavonoids (fruits and vegetables), catechins (tea and wine), benzene derivatives, caffeine, thiram (agricultural fungicide), and quinolones (antibiotics) *(26,27)*. Extensive experimental and epidemiologic studies now under way should clarify the role of these products in the etiology of infant leukemia.

Interindividual pharmacogenetic differences in the metabolism of these products might be important in the genetic susceptibility to infant leukemia. A low NADPH:quinone oxidoreductase (NQO1) activity has been correlated with *MLL*-gene-rearranged leukemia *(28)*. The quinone moiety is shared by many topoisomerase II-inhibiting products. Glutathione S transferase (GST) isoform polymorphisms may also play a role in leukemogenesis. The frequency of single and double GST gene deletions was higher in the parents of infants with leukemia than in the general population *(24)*.

It might be that different types of leukemia have different etiologies; for example, *MLL*-gene-rearranged infant leukemia might arise from entirely different exposures to mutagens during pregnancy than encountered by the mothers of children with common ALL developing at the peak ages of 2–5 yr, in which infections could play a leading role *(29)*. However, it should be stressed that such explanations are highly speculative and that the cause of (infant) leukemia remains to be elucidated.

3.3. Risk Factors

Constitutional chromosomal abnormalities account for a very small proportion of infant leukemias. Trisomy 21, especially, is a predisposing factor in the development of infant leukemia, whereas Down's syndrome increases the risk for leukemia development 20-fold *(27,30)*. Infants with Down's syndrome and leukemia almost always have ANLL, especially the megakaryoblastic type FAB M7 *(31)*. Down's syndrome does not predispose to ALL in the first year of life, as illustrated by the Children's Cancer Group (CCG) series of 234 infants with ALL, none of whom had this syndrome *(13)*.

Most etiologic studies of childhood leukemia have focused on the combined age categories and give no specific information regarding infants. Hence, the roles of space-time clustering, population mixing, infectious agents and vaccinations *(19,29,32–34)*, socioeconomic status and urbanization *(35)*, ionizing radiation, nonionizing radiation/electromagnetic fields *(36)*, maternal history of fetal loss, and breast feeding *(37)* in leukemia induction are not clear for infants *(27)*. The number of infants in one of the studies describing seasonal variations in the onset of childhood leukemia is too low to permit conclusions on infant leukemia *(38)*. The contribution of the radioactive fallout from the Chernobyl accident to a transient increase in infant leukemia is controversial *(34,39,40)*.

Shu et al. *(41)* showed a relationship between maternal marijuana use and infant leukemia, as well as a dose-dependent relationship between maternal alcohol consumption during pregnancy and the risk of infant ANLL, especially the M1 and M2 morphologic subtypes. It was postulated by the authors that ethanol may induce microsomal enzymes (e.g., cytochrome P450) that are involved in precarcinogen metabolism. There was only a modest relationship between maternal alcohol consumption and the risk of infant ALL and none involving paternal alcohol consumption. Remarkably, in the same study, maternal smoking during pregnancy was negatively (but weakly) associated with the risk of infant leukemia. The association between maternal smoking and the risk of childhood leukemia in older age categories is highly controversial *(27)*. Paternal smoking 1 mo before pregnancy was suggested to be related to a slight increase in the risk of infant ALL, whereas income, age, and education of the parents showed no relationship *(41)*.

Yeazel et al. *(42)* described an increased risk of both ALL and ANLL diagnosed before the age of 2 yr, in infants with a high birth weight, in concordance with other studies reporting the same relationship for older children with ALL *(27)*. Ross et al. *(43)* suggested that high levels of insulin growth factor-1 play a role in this association. Cancer history in parents was also found to be associated with an elevated risk of infant leukemia, especially ANLL, whereas a family history of autoimmune disease was not *(44)*.

Again, it is important to realize that all relationships between the described factors and the etiology of infant leukemia are weak or modest, implying the existence of other, still-unidentified causative factors.

4. INFANT ACUTE LYMPHOBLASTIC LEUKEMIA

4.1. Clinical Presentation and Biology

Compared with older children who have ALL, infants with this disease present with a high leukocyte count and an increased frequency of hepatosplenomegaly and central nervous system (CNS) involvement (Table 1). Two-thirds of the infants present with a leukocyte count $>50 \times 10^9$/L, and in about half of this group the count is $>100 \times 10^9$/L. In most studies, and when the series in Table 1 are combined, about 15% of infant ALL cases have CNS involvement at diagnosis. This percentage was about 25% in several series *(4,14)*. Thrombopenia $<50 \times 10^9$/L was found in about 60% of all infants with ALL, and enlarged testes were seen in 14 of 106 male infants *(13)*. The mediastinum was markedly enlarged in <5% of infant ALL cases *(13)*.

4.1.1. Immunophenotype

The immunophenotypic characteristics of infants with ALL are summarized in Table 2. About two-thirds of the cases can be classified as immature CD10- negative B-lineage precursor, described by different authors as pro-B, early pre-B, or pre- pre-B. The remaining group mainly consists of common/pre-B cases. Mature B-lineage ALL is an exceptional finding *(15)*, whereas T-lineage ALL was diagnosed in only 22 (4%) of the 505 cases described in Table 2. Infant ALL cells are more likely to express myeloid-associated antigens *(45–47)*. Some cases are difficult to classify because they lack lineage-specific markers or express both myeloid and lymphoid markers (mixed or biphenotypic leukemia) *(13–15)*. The myeloid marker myeloperoxidase is frequently expressed in infant ALL cells *(48)*. These data suggest that infant ALL arises from an immature precursor cell that is not fully committed to lymphoid differentiation. Intraclonal switch from B-lineage to monocytic lineage during therapy has been described in some infant leukemias *(49)*.

Table 2
Immunophenotypic Characteristics of Infant ALL at Diagnosis

Characteristic	*No. of patients*[a]	*Reference*
B-lineage/T-lineage/ unclassified (percentage of cases with T-lineage markers)	67:4:11 (5)	*14*
	27:1:5 (3)	*15*
	25:5:24 (9)	*13* (CCG-107)
	88:3:8 (3)	*13* (CCG-1883)
	21:2:0 (9)	*18*
	99:3:2 (3)	*4*
	21:1:6 (4)	*16*
	70:3:9 (4)	*46*
CD10-negative/ CD10-positive (percentage of CD10-negative cases)	45:23 (66)	*14*
	19:6 (76)	*15*
	42:13 (76)	*13* (CCG-107)
	56:41 (58)	*13* (CCG-1883)
	10:13 (43)	*18*
	15:9 (63)	*7*
	62:42 (60)	*4*
	16:12 (57)	*16*
	40:32 (56)	*46*

[a]Percents are in parentheses.

4.1.2. Cytogenetics

Cytogenetic analysis of infant ALL cells reveals abnormalities in about three-fourths of all patients. However, these percentages are based on the number of samples for which successful cultures were obtained and not the total population. Thus, the percentage of cases with chromosomal abnormalities detected by cytogenetic analysis is lower than Table 3 suggests. Cytogenetic abnormalities that occur relatively frequently in older children, such as the Philadelphia translocation t(9;22), t(1;19), hyperdiploidy, and t(12;21), resulting in the *TEL/AML1* fusion product, are detected only rarely in infant ALL.

The most common chromosomal aberrations in infant ALL are translocations involving chromosome band 11q23. By karyotyping, 11q23 abnormalities are detected in about 50% of all infants with ALL with succesful cytogenetic studies. t(4;11)(q21;q23), the most common translocation, is found in about 60–70% of the cases with a 11q23 rearrangement. t(11;19)(q23;p13) is found in about 15% of cases and t(9;11)(p22;q23) in a still lower frequency. Many other 11q23 partner chromosomes have been reported, all occurring at a very low frequency. Translocations affecting the 11q23 region involve the *MLL* gene, whereas most ALL cases with deletions or inversions of 11q23 do not involve this gene *(50)*. The latter abnormalities are especially common in older patients with ALL but occur infrequently in infants with ALL, who most often have balanced translocations. Thus, in infant ALL cases with 11q23 abnormalities, the *MLL* gene is almost always rearranged *(50)*.

Table 3
Chromosomal Abnormalities in Infant ALL at Diagnosis

Patients with chromosomal abnormalities		*Patients with 11q23 abnormality*		*11q23 abnormality*				
No./Total	*%*	*No./Total*	*%*	*Type*	*No./Total*	*%*	*Technique*	*Reference*
47/64	73	27/64	42	t(4;11) other	20/27 7/27	74 26	Cytogenetics	*14*
29/33	88	19/33	58	t(4;11) t(11;19) other	13/19 3/19 3/19	68 16 16	Cytogenetics	*15*
41/59	69	29/59	49	t(4;11) t(11;19) t(9;11) other 4/29	17/29 4/29 4/29	59 14 14 14	Cytogenetics	*4*
7/10	70	4/10 6/8	40 75	t(4;11) ?	4/4 —	100 —	Cytogenetics Southern blot	*18*
9/15	60	6/15	40	t(4;11)	6/6	100	Cytogenetics	*16*
39/48	81	27/48	56	t(4;11) t(11;19) other	15/27 7/27 5/27	55 26 19	Cytogenetics	*17*
40/56	71	28/56	50	t(4;11) t(11;19) other	21/28 4/28 3/28	75 14 11	Cytogenetics	*66* (CCG-1883)
28/39	72	17/39	44	t(4;11) other	12/39 5/39	31 13	Cytogenetics	*69* (CCG-107)
?	—	78/96	81	?	—	—	Southern blot	*56*
?	—	50/96	52	t(4;11) t(11;19) other	36/50 7/50 7/50	72 14 14	Cytogenetics	
?	—	18/20	90	?	—	—	Southern blot	Beverloo (in *51*)
?	—	29/40	73	?	—	—	Southern blot + RT-PCR (*MLL/AF4*)	*52*
?	—	19/40	48	?	—	—	Cytogenetics	

Abbreviations: CCG, Children's Cancer Group; RT-PCR, reverse transcriptase polymerase chatin reaction.

Cytogenetic analysis detects 11q23 abnormalities in about 50% of infant ALL cases, but this percentage rises to 70–90% when molecular techniques, such as Southern blotting, polymerase chain reaction (PCR), or fluorescence *in situ* hybridization (FISH), are used (Table 3) *(51–56)*. Conventional cytogenetic analysis underestimates the number of cases with rearranged *MLL* genes, mainly because of technical problems with obtaining metaphases in culture. Southern blotting has the advantage of diagnosing all possible *MLL* gene rearrangements, but high numbers of cells are necessary and the partner gene cannot be detected. Reverse transcriptase (RT)-PCR will also detect more positive cases but will do so only when the partner gene is well defined. Most *MLL* gene rearranged cases will be detected if RT-PCR for the well-known t(4;11), t(9;11), and t(11;19) fusion transcripts is performed. Three recently described PCR techniques should facilitate detection of *MLL* gene rearrangement. These are the multiplex RT-PCR analysis, which simultaneously detects known partner genes in 11q23 translocations *(57)*, and the panhandle PCR method, which identifies translocations with unknown partner genes *(58,59)*. The split-signal FISH method can detect all types of *MLL* gene translocations in a single FISH experiment *(60)*, requiring a very low amount of material.

4.1.3. Function of the *MLL* Gene

The structure of the *MLL* gene, also called *ALL-1* or *HRX*, shows a number of domains that are important for DNA binding and transcriptional control *(61)*. The exact function of the normal *MLL* gene is unknown, as is the possible leukemogenic role of *MLL* gene abnormalities. The transcription factor function of *MLL* is probably disrupted by translocations, which break the gene between the two DNA binding regions. The DNA binding motifs of the *MLL* gene (as well as the transactivation activity of the partner gene *ENL* at chromosome 19p13) are required for in vitro immortalization of murine myeloid cells *(62)*. The chimeric transcript of *MLL-LTG19* in a cell line carrying t(11;19) contributes to cell proliferation and

malignant transformation *(63)*. Double knockout of the *MLL* gene blocks hematopoietic differentiation *(64)*. Knockin studies showed that mice expressing the *MLL-AF9* fusion gene develop ANLL *(65)*. However, in the most frequently occurring t(4;11)-positive infant ALL, it is still unclear whether the fusion protein encoded by the derivative 11 chromosome or the derivative 4 chromosome is the oncogenic protein. The ubiquitous presence of the derivative 11 fusion protein in ALL cases with many other partner genes suggests that the derivative 11 fusion protein contributes to leukemogenesis. Suggestive evidence that the clinical outcome of infants with *MLL* rearrangements might differ depending on the partner gene indicates that the fusion partner might play an important role in leukemogenesis and response to therapy. Sequence analysis of the *AF-4* gene suggests a role in transcriptional regulation *(61)*.

4.4.1. Relationships Among Biologic Factors

The presence of *MLL* gene rearrangements, the absence of CD10, the coexpression of myeloid antigens, and a high leukocyte count are highly correlated with each other, and all are inversely related to the age of the infant *(47,52,56,66)*. About 90% of CD10-negative infant ALL cases carry *MLL* rearrangements, compared with 20% of their CD10-positive counterparts *(47,52,53)*. From another point of view, 90% or even 100% of the *MLL* rearranged cases have been described as CD10-negative, whereas 80% of the germline *MLL* cases are CD10-positive *(52,53,66)*. Two-thirds of the infants with *MLL* rearranged ALL are younger than 6 mo of age, contrasted with only one-fourth of the germline *MLL* cases *(53,66)*. The *MLL* rearranged cases have significantly higher leukocyte counts *(47,56,66)* and express myeloid antigens more frequently than do their *MLL* germline counterparts *(46,47)*. Thus, lymphoblasts from infants with ALL are typically CD10-negative, have rearranged *MLL* genes with myeloid antigen coexpression, and present with a high leukocyte count. About one-third of all infants with this disease lack these features.

4.1.5. Biology and Prognosis

The poor prognosis of infant ALL has been associated with the following factors in univariate analysis: age younger than 3 or 6 mo *(4,13,14,17,47)*, organomegaly *(13)*, CNS involvement *(4)*, a high leukocyte count *(4,13,14,17,18,67,68)*, CD10 negativity *(4,13,17,18,55)*, myeloid antigen expression *(45,47)*, *MLL* gene rearrangement *(4,13,47,52–56,69)*, and d-14 bone marrow response to therapy *(13)*. Gender was not a prognostic factor *(4,14,17,18,53)*. The above results represent a number of different protocols, although it can be concluded that the event-free survival (EFS) of infants younger than 6 mo of age ranges from 10 to 30%, compared with 40–60% for infants 6–12 mo of age. The EFS for CD10-negative infant ALL is about 20–30%, compared with about 50–60% for its CD10-positive counterpart. The EFS rates for *MLL* rearranged vs germline *MLL* cases are 5–25% and 40–60%, respectively. In the studies of the Children's Cancer Group (CCG), clinical outcome was especially poor for patients with the t(4;11) but not other *MLL* gene rearrangements, although too few cases were included in the latter category to allow definitive conclusions *(13)*. Irrespective of how low- and high-risk groups of infants are defined, the prognosis of the high-risk group is extremely poor, whereas the outcome for so-called low-risk groups is still considerably worse than matched counterparts in the general population of childhood ALL patients.

Because the risk factors mentioned above are closely related, it is very difficult to determine the factors with independent prognostic significance. Multivariate analyses to resolve this issue have not been performed or were hampered by small numbers of cases or by the fact that cytogenetic data were not available in many of the cases. In fact, only two studies have performed a reasonably valid multivariate analysis. The data of Reaman et al. *(13)* show that t(4;11) status rather than CD10 status is the more important determinant of outcome (the impact of age and leukocyte count was not entirely clear). Day-14 bone marrow response remained of prognostic relevance after adjustment for *MLL* status: the reverse analysis was not done. The Berlin-Frankfurt-Münster (BFM) Group *(4)* showed that the clinical response to 1 wk of therapy with prednisone was the strongest independent predictor of outcome in infant ALL. Age and leukocyte count also had independent prognostic value, but *MLL* gene rearrangement lost its predictive strength in the multivariate model, which included prednisone response. The ongoing collaborative prospective trials in infant ALL described later in this section should clarify which factors have the strongest independent impact on treatment outcome.

4.1.6. Biology and Drug Resistance

Some studies have shown that the factors age, immunophenotype, and *MLL* rearrangement reflect or cause changes in cellular drug resistance factors, providing insight into why infants have such a poor prognosis. It was shown that leukemic cells from infants with 11q23-rearranged ALL cells (1) grew better on stromal cell layers in vitro *(70)*; (2) had a higher leukemic cell recovery when inoculated into SCID mice *(71)*; and (3) were more resistant to cell death resulting from serum deprivation in vitro *(72)* when compared with cells from other children with ALL. Infant ALL cells were significantly more resistant in vitro to prednisolone and L-asparaginase than cells from older children *(73)*, in concordance with the finding of the BFM group that infants with ALL more frequently show a poor response to prednisone than do older children with ALL *(3,4)*. In vitro and in vivo resistance to these drugs is a strong adverse prognostic factor *(4,74–77)*. Of further interest was the finding that the leukemic cells of infants with ALL were significantly more sensitive to cytarabine in vitro than cells from older children *(73)*. In addition, precursor B-lineage ALL samples that lacked CD10 expression were also significantly resistant to prednisolone and L-asparaginase but showed significant sensitivity to cytarabine compared with the same lineage of cells expressing CD10. It has been suggested that the use of high-dose cytarabine after induction therapy might benefit infants with ALL *(18)*. The survival of adults with early B-cell ALL, with or without 11q23 rearrangements, has improved with the introduction of high-dose cytarabine/mitoxantrone consolidation blocks *(78)*.

Pharmacokinetic resistance probably plays no important role in infant leukemia because infants do not show increased clearance of drugs. However, inadequate drug dosage adjustments may lead to undertreatment.

Table 4
Treatment Results for Infant ALL

No. of patients	*Complete remission* No./total	%	*Outcome (%)*	*Relapse or 2nd malignancy (%)*	*Death in remission* No./total	%	*Protocol and reference*
23	22/23	96	4-yr EFS, 54± 11	6/23 (26) 3 BM, 2 BM + CNS, 1 2nd malig	3/23	13	DFCI Consortium 73-01, 77-01, 81-01 *(18)*
82	76/82	93	4-yr EFS, 27 ±6	50/82 (61) 35 BM, 8 BM + CNS, 5 CNS, 1 BM + CNS + testis, 1 BM + testis (3 BMT pts censored)	4/82	5	POG 8493 *(14)*
33	31/33	94	5-yr EFS, 17 ± 8	24/33 (73) 13 BM, 3 BM + CNS, 3 CNS, 4 testis, 4 CNS + testis (1 BMT pt censored)	1/33	3	POG pilot *(15)*
99	94/99	94	4-yr EFS, 33 ± 5	59/99 (59) 35 BM, 7 BM + CNS, 8 CNS, 4 BM + testis, 2 testis, 3 other	2/99	2	CCG-107 *(13)*
135	131/135	97	4-yr EFS, 39 ± 4	74/135 (55) 55 BM, 7 BM + CNS, 4 CNS, 4 BM + testis, 2 testis, 2 other	5/135	4	CCG-1883 *(13)*
105	100/105	95	6-yr EFS, 43 ± 5	50/105 (48) 26 BM, 10 BM + CNS, 9 CNS, 3 testis, 1 BM + testis, 1 2nd malig.	4/105	4	ALL-BFM 83, 86, 90 *(4)*
28	24/28	86	4-yr EFS, 43 ± 19	11/28 (39) 7 BM, 3 BM + CNS, 1 CNS	1/28	4	EORTC-CLCG 58831, 58832 *(16)*
88	81/88	92	5-yr EFS, approx. 25	44/88 (50) 23 BM, 14 CNS, 6 BM + CNS, 1 testis	12/88	14	MRC-UKALL VIII, X, pilot *(17)*

Abbreviations: EFS, event-free survival; BM, bone marrow; BMT, bone marrow transplant;CNS, central nervous system; DFCI, Dana-Farber Cancer Institute; POG, Pediatric Oncology Group; CCG, Children's Cancer Group; BFM, Berlin-Frankfurt-Munster; EORTC-CLCG, European Organization for Research and Treatment of Cancer - Children's Leukemia Cooperative Group; MRC-UKALL Medical Research Council, United Kingdom, ALL trials.

4.2. Treatment

4.2.1. Results and Failures

Whereas the overall cure rate for childhood ALL has risen to 70–80% in most contemporary treatment programs, progress in the treatment of infant ALL has remained behind. Table 4, which summarizes the results of recently published studies by different groups, shows an overall EFS rate of 30–40%. The complete remission rate is about 94% in most studies, close to the figures attained in older children with ALL. Toxicity does not pose a major problem after remission induction. (Approximately 4% of the infants die from drug-related toxic effects.) The major cause of treatment failure is relapse: in the combined series shown in Table 4, 318 of 593 patients (54%) experienced a relapse, which involved the bone marrow in 80% of cases, the central nervous system (CNS) in 30%, and the testes in 8%. Most relapses occur early. In the study of Lauer et al. *(15)*, the median time to relapse was only 39 wk. In the BFM studies, two-thirds of all relapses in the prednisone poor-responder group and one-third of the relapses in the prednisone good-responder group took place within 6 mo after diagnosis during intensive treatment *(4)*. In the largest published series of the CCG, two-thirds of all relapses occurred less than 1 yr after end of induction therapy. About 80% of patients who experienced a relapse died of disease *(13)*. These data show that early relapse is the major cause of death in infant ALL: 6% of all patients will not obtain a complete remission, 4% will die of toxicity in remission, and 45–50% will die from relapse.

4.2.2. Comparison of Treatment Protocols and Results

The outcome of infant ALL has improved with the most recently reported treatment protocols, including those of the BFM *(4)*, CCG *(13)*, Dana-Farber Cancer Institute Consortium (DFCI) *(18)*, and Pediatric Oncology Group (POG) *(14)*, although contributions from nontherapeutic factors cannot be ruled out in the context of comparisons with historical data. Intergroup comparisons of treatment protocols and outcome are difficult because most protocols differ in many details, the number of patients with high-risk characteristics may differ,

some reports combine the results of several consecutive protocols, and the sizes of the study populations are relatively low, even in the larger trials. Nevertheless, the results of such comparisons can be informative with regard to the most useful treatment strategies.

Table 4 shows that a study performed by several POG institutions resulted in a 5-yr EFS of only 17% *(15)*. The backbone of this regimen was the so-called intensive alternating drug pairs introduced after remission induction. Unlike other protocols, this regimen did not contain dexamethasone, high-dose methotrexate (MTX), high-dose cytarabine (ara-c), cyclophosphamide, or ifosfamide. L-Asparaginase was used in the induction phase only, and no craniospinal irradiation was given. In another POG study (8493) recently reported by Frankel et al. *(14)*, the EFS rate of 27% also seems lower than the results of other study groups. This protocol lacked dexamethasone, L-Asparaginase, anthracyclines, high-dose ara-c, and high-dose MTX, in contrast to the protocols of most other groups. It also lacked CNS irradiation.

Protocols of the Medical Research Council United Kingdom, ALL (MRC UKALL) study specified high-dose MTX dose and high-dose ara-c, but not dexamethasone, cyclophosphamide, or ifosfamide *(17)*. L-Asparaginase was administered only in the induction phase. The overall EFS rate was 25%, lower than that reported by other groups. The DFCI Consortium has intensified its treatment protocols since 1985, leading to a significant improvement in treatment results (4-yr EFS, 54%) *(18)*. The main departure from the historical control series was the introduction of a postinduction intensification phase including high-dose MTX, high-dose ara-c, L-Asparaginase, vincristine, and 6-mercaptopurine. Dexamethasone (except for an investigational window in part of the treatment plan), cyclophosphamide or ifosfamide, and epipodophyllotoxins were all excluded from the DFCI protocol, beginning in 1985. Cranial irradiation was administered at the age of 1 yr. The limited number of infants enrolled in these studies mandates confirmation of the high EFS rate by other groups using similar therapy.

The BFM study group did not treat infants on a separate protocol but within programs for the general population of ALL patients. Since 1983, BFM investigators have stratified patients according to the early prednisone response and presenting leukemic cell burden, resulting in diverse treatments for the infant subgroup. Infants were overrepresented in the higher-risk arms because of their higher leukemic cell burden and the high number of patients with a poor response to prednisone. The overall EFS rate for infants, 43%, is among the highest of reported results. A small study of the European Organization for Research and Treatment of Cancer-Children's Leukemia Cooperative Group *(16)*, using a slightly modifed BFM regimen, also resulted in a 43% EFS. In both studies, cranial irradiation was given to a subgroup of the the patients.

The most recently completed infant study of the CCG (1883) yielded a 39% EFS rate, comparable to the results reported by BFM and DFCI investigators and not significantly different from the 33% EFS rate in the earlier CCG-107 study *(13)*. These outcomes were better than historical CCG control data based on less intensive systemic therapy but including cranial radiotherapy. Importantly, the CNS relapse rates in the more recent 107 and 1883 studies, which relied on intensive systemic chemotherapy and intrathecal therapy, without irradiation, were no higher than those associated with radiation. Major differences between the two modern CCG protocols and historical controls were (1) inclusion of high-dose ara-c, cyclophosphamide, and more L-asparaginase in the consolidation and reconsolidation phases of the more recent trials; (2) the use of prednisone instead of dexamethasone; and (3) omission of 6-thioguanine in the reinduction/reconsolidation phase in the 1883 study.

An important finding was that cranial irradiation and intensive chemotherapy combined with intrathecal therapy result in the same CNS relapse rate, even in patients with CNS involvement at initial diagnosis *(13)*. Additional evidence for the relative inefficacy of cranial irradiation in preventing CNS relapse can be deduced from Table 4: studies including cranial irradiation do not show a lower CNS relapse rate. In particular, high-dose MTX, high-dose ara-c, dexamethasone, and intrathecal therapy seem good candidates for CNS-directed therapy.

Another possible conclusion from a comparison of historical controls, intensive POG protocols, and more recent protocols of the CCG, BFM, and DFCI is that intensive postinduction chemotherapy and the use of high-dose ara-c, high-dose MTX, L-asparaginase, dexamethasone, and cyclophosphamide/ifosfamide are helpful in preventing early bone marrow relapses. In the context of the low incidence of infant ALL, large international collaborative studies are needed to study new therapies for this disease. Two large collaborative efforts—a joint study of the CCG and POG and the international Interfant-99 study involving many European and several non-European groups—are currently analyzing the efficacy of intensified therapy for infant ALL.

4.2.3. Bone Marrow Transplantation

The data on autologous stem cell transplantation in infant ALL are limited to case reports, precluding conclusions as to efficacy *(16,17,79)*. Similarly, the role of allogeneic bone marrow transplantation (BMT) in infant ALL is unclear and debatable. No proper randomized studies have been performed comparing allogeneic BMT with continued chemotherapy, and the published data are scant and selective. Ferster et al. *(16)* report one case of succesful BMT, Chessells et al. *(17)* report two toxic deaths and one relapse among three BMT cases, and Pirich et al. *(80)* note four leukemia-free survivors out of seven transplanted patients. Reaman et al. *(13)* mention that 12 patients on CCG-1883 underwent BMT in first remission: only 2 survived event-free; 5 died in remission and 5 experienced a posttransplantation relapse. Data from the European Group for Blood and Marrow Transplantation (EBMT; personal communication, September 1997) show a 40% leukemia-free survival rate for 76 infants with ALL who underwent BMT. There were no significant differences in outcome among HLA-identical, matched unrelated donor, and non-HLA-identical related donors. These results are selective, reflecting only those infants who have survived to the time of transplantation. Altogether, the limited data do not encourage the use of BMT on a large scale in infant ALL. Controlled studies are needed to determine the usefulness of BMT in this vulnerable group of patients. The efficacy of BMT

is being studied in a collaborative POG/CCG study. BMT in the Interfant-99 study is restricted to infants with a poor response to prednisone, which uniformly indicates a dismal prognosis.

5. INFANT ACUTE NONLYMPHOBLASTIC LEUKEMIA

5.1. Clinical Presentation and Biology

Infant ANLL is characterized by a high leukocyte count, hepatosplenomegaly, chloroma (leukemia cutis), and a higher incidence of CNS involvement at diagnosis compared with that in older children with this disease *(6,67,81,82)*. The FAB subtypes M4/M5 and M7 are significantly more often found in infants than in older children with ANLL *(67,81,83)*. The percentage of M4/M5 cases in infant ANLL ranges from 58 to 84%, whereas the percentage of M7 cases is much lower, about 10%, possibly because Down's syndrome cases, which tend to have a high incidence of M7 ANLL, were excluded in some reports *(7,67,81,84,85)*.

5.1.1. Cytogenetics

Rearrangements of chromosome 11q23 are the most frequent abnormality in infant ANLL. With routine cytogenetic analysis, about 40–50% of the cases have an abnormality affecting the q23 region of chromosome 11 *(84,86–89)*. With molecular techniques, *MLL* gene rearrangements are detected in 58-66% of cases *(84,85,90)*. These percentages are much higher than those in the overall population of childhood ANLL patients, 15–20% of whom carry this abnormality *(31)*. The 11q23 rearrangement is closely linked to the M4/M5 FAB subtype and a high leukocyte count. More than 90% of infants with monoblastic or myelomonoblastic (M4/M5) ANLL have this abnormality, contrasted with <10% of other infants with ANLL *(84,85)*. t(4;11), t(9;11), and t(11;19) are the most common translocations involving 11q23 in infant ANLL, although many other partner chromosomes can be involved *(31)*. Hilden et al. *(91)* studied infant ANLL cells with a monoclonal antibody directed against the human homolog of the rat NG2 chondroitin sulfate proteoglycan molecule. All 13 NG2-positive cases had *MLL* gene rearrangement, but 13 of 24 NG2-negative cases also had an *MLL* rearrangement.

t(1;22)(p13;q13) occurs in only 1–4% of childhood ANLL series overall *(92,93)* but in 6–28% of infant ANLL cases *(94,95)*. When only ANLL-M7 cases are considered, t(1;22) is detected in 17% of children but in 45% of infants *(96)*. The t(1;22) abnormality is described almost exclusively in infants with ANLL-M7 who lack Down's syndrome *(31,92–95,97)* and is associated with a low leukocyte count, myelofibrosis, and organ infiltration *(31,86,94)*.

5.1.2. Down's Syndrome and Transient Leukemoid Reaction

Down's syndrome is associated with a 20-fold increased risk for leukemia in general and a 500-fold-higher incidence of ANLL-M7, *(31,98)*, which frequently presents with myelodysplasia or transient leukemia. Often trisomy 8 without t(1;22) is found in the M7 (megakaryoblastic) leukemic cells *(31,99,100)*. This form of ANLL has an unfavorable prognosis in children without Down's syndrome, but is prognostically neutral in children with Down's syndrome *(97,99)*. Children with Down's syndrome respond very well to chemotherapy *(99,101)*, and their leukemic cells are significantly more sensitive to anthracyclines and ara-c than are the cells from other children with ANLL *(102,103)*.

Congenital leukemia or leukemias in very young infants should be discriminated from a transient myeloproliferative disorder (TMD) or transient leukemoid reaction. The latter occurs almost exclusively in neonates with Down's syndrome or mosaicism for trisomy 21, although it has also been described in other neonates *(83,86,100,104)*. It is suggested that congenital leukemia and transient leukemia can be distinguished from each other by in vitro colony formation assays *(105)*. [Cases with TMD had normal cell growth patterns in granulocyte/macrophage colony-forming unit (GM-CFU) assays.] TMD disappears within weeks to months without antileukemic treatment although Homans et al. *(106)* found a mortality rate of 11% during the postnatal period from secondary causes. Moreover, about 30% of patients will subsequently develop acute leukemia after months to years, especially the ANLL-M7 subtype. These figures indicate that adequate monitoring of infants with Down's syndrome and TMD is advisable. Because infants with Down's syndrome and ANLL-M7 have an excellent prognosis, newborns with TMD should receive only aggressive supportive care, with antileukemic therapy reserved until overt leukemia is apparent.

5.1.3. Prognosis

Whether the prognosis of infants differs from that of older children with ANLL is controversial. Some authors *(11,107)* report a poorer outcome in infant ANLL, whereas others *(8,81,108–110)* suggest a comparable outcome. Comparison of these studies is hampered by differences in treatment and definition of patient categories. In the BFM series, for example, young ANLL patients were defined as being under 2 yr of age *(81)*; in the French study in which a poorer outcome for infants was found, only 1 of 19 infants had BMT, compared with 32 of 130 older children with ANLL *(11)*. In general, the currently available data suggest that infants with ANLL do not have a poorer outcome than older children with ANLL. This does not imply that infant ANLL has a better outcome than infant ALL; to the contrary, outside the infant population the outcome of ANLL is poor compared with that in ALL.

Pui et al. *(7)* showed that among infants with ANLL, the leukocyte count and male sex were adverse prognostic factors, but the presence of an 11q23 abnormality lacked prognostic impact, a finding confirmed by Satake et al. *(84)*. The 5-yr EFS rates of *MLL*-rearranged ANLL cases were 44 and 42%, respectively, in these two studies, compared with 31% for the *MLL* germline cases in both studies. Similarly, Sorensen et al. *(85)* found no difference in complete remission rates between infants with ANLL, with or without *MLL* gene rearrangement. The prognosis of ANLL-M7 with t(1;22) appears to be poor *(31,86,93,95)*, although no large series with these patients have been published. As mentioned earlier, infants with Down's syndrome and ANLL-M7 have a relatively good prognosis.

5.2. Treatment

In contrast to ALL, there are no separate protocols for the treatment of infant ANLL, reflecting the similar prognoses of infants and older children with this disease. For the detailed outlines and results of ANLL treatment protocols, the reader is

referred to the chapter on childhood ANLL in this book. The suggestion that infant monoblastic leukemia is particularly responsive to the epipodophyllotoxins *(86)*, requires further confirmation. The value of allogenic BMT has been established in childhood ANLL in general, but there are only scant data on the results of this procedure in infant ANLL *(79,111)*. BMT is not indicated for infants (and older children) with Down's syndrome and ANLL because of their good outcome with chemotherapy alone.

6. LATE EFFECTS OF TREATMENT

The late effects of treatment for infant leukemia are poorly understood, mainly because substantial numbers of infants did not survive until recently. In studies reported to date, learning disabilities and developmental delays were identified in 9 of 11 and 2 of 4 irradiated infants *(16,18)*. Obesity and short stature were found in 3 and 2 of 11 irradiated cases, respectively. Asymptomatic echocardiographic abnormalities and stable congestive heart failure have been reported in single cases *(16,18)*.

In 30 nonirradiated infants who were treated with high-dose MTX as CNS-directed therapy, the neurodevelopmental outcome was normal *(112)*. Frankel et al. *(14)* reported on one patient with a severe developmental disorder among 18 infants who were neither irradiated nor transplanted and remained in complete remission. Woolfrey et al. *(111)* showed that in infants younger than 2 yr with ANLL or myelodysplastic syndrome, neurologic development was normal after a conditioning regimen with cyclophosphamide in combination with either busulfan or total-body irradiation (TBI), the latter having an adverse effect on growth. As treatment becomes more effective for infants with leukemia, it will be important to incorporate prospective studies of late effects into all new protocols.

7. OTHER CONTROVERSIAL ISSUES

Apart from BMT and prognostic factors, other controversial issues in infant ALL are how to adjust the drug dosage and whether all infants with leukemia should receive the same therapy.

7.1. Drug Dosage Adjustment

A persistent problem is the rules for drug dosage adjustment in infants with leukemia or cancer in general. A number of factors have to be taken into account *(24)*. The total-body water content decreases from 75% at birth to 60% at 1 yr, and the percentage of extracellular water also decreases with age *(113)*. Drugs bind less avidly to serum proteins in newborns than in adults, leading to a higher unbound active fraction of drugs in infants *(114)*. The lower activity of P450 enzymes in infants *(115,116)* can lead to reduced cytotoxic effects as well as increased cytotoxic effects, depending on whether drugs are activated or inactivated by these enzymes. Drugs that are cleared by the kidneys can have increased systemic exposures and pharmacologic effects in young infants because renal tubular and glomerular function reach adult levels by about 6 mo of age *(24)*. The volume of the CNS relative to body surface area or body weight is larger in children compared to adults. Therefore, intrathecal chemotherapy should be calculated on age and not on body surface to avoid undertreatment of young children *(117)*. The ratio of body weight to body surface is lower in infants than in older children and adults, which implies that if dosages are calculated on body weight, infants will receive lower amounts of drugs than do older children.

A study of seven infants with ALL aged 3–12 mo showed no decreased clearance of MTX compared with results in older children *(119)*. It has been suggested that infants show decreased ara-C clearance after high-dose therapy with this agent because of poorer conversion of ara-C to ara-U relative to that in older children with ALL, although a direct comparison of infants with older children was not presented *(120)*. Others have not found a difference in ara-C clearance between infants and older children *(118)*. At the moment, there is no standard procedure for drug dosing in infants. Current protocols rely on arbitrary calculations based on body weight, body surface area, or one of these with a correction for age. Boos et al. *(121)* showed that a dose calculation based on two-thirds of the dose by body weight resulted in decreased steady-state levels of etoposide, as well as decreased toxicity. However, etoposide clearance by body weight or body surface area did not differ between children and infants 3 to 12 mo of age. A small study by McLeod et al. *(118)* suggested that dosages of epipodophyllotoxins and ara-c based on body surface area would lead to similar exposures in infants and adults, whereas for doxorubicin, the same effect was more likely to be achieved by dosing according to body weight. Thus, pharmacokinetic studies together with toxicity measurements are urgently needed in infants with leukemia or other types of cancer.

7.2. Identical Treatment for All Infants with Leukemia?

The question of whether all infants should receive identical therapy is difficult to answer. First, it should be determined whether infant ANLL and ALL could be treated according to one protocol and second, whether different protocols should be used for subcategories of infant leukemia. So far, ANLL is treated identically in infants and older children, whereas infants with ALL are usually treated according to high-risk protocols for all patients or protocols designed specifically for infants. ALL and ANLL in infants are alike in some regards (both arise in a primitive hematopoietic cell possessing both lymphoid and myeloid characteristics, as well as *MLL* gene rearrangements) but differ markedly in others. [Leukemic B-cell precursors predominate in ALL cases, whereas a range of FAB subtypes can be identified in ANLL. Also, Down's syndrome patients with M7 leukemia and M7 cases with a t(1;22) reflect disease subtypes not found in infant ALL.] Thus, any consideration of identical therapies for infant ALL and ANLL should probably be limited to *MLL*-gene rearranged cases.

Some investigators have suggested that intensive therapy for infant ALL should be limited to the high-risk group. However, it is still unclear which infants actually warrant a high-risk classification, and even the so-called low-risk infant ALL cases have a poorer outcome than matched cases with an older age. It is often assumed that the *MLL*-gene rearranged group should be regarded as the high-risk group, but, as argued above, these patients do not constitute a homogenous group. Dordelmann et al. *(4)* showed that *MLL*-gene rearranged cases with a good response to prednisone do not have a poor outcome. Also, in daily practice the *MLL* gene status is unknown in a considerable number of patients, and the technique for demonstrateing *MLL*

gene status can produce highly variable results. Thus, large collaborative studies are needed to answer this question and whether infants should be treated on standard ALL and ANLL protocols or assigned to separate, experimental protocols.

8. CONCLUSIONS AND PERSPECTIVES

The origins of infant leukemia are largely unknown. Both epidemiologic and basic molecular studies are necessary to unravel the etiology and pathogenesis of this disease. The clinical outcome of infant ANLL is not worse than that of older children with ANLL, but treatment for all children with ANLL needs to be improved. Infant ALL shows a highly unfavorable outcome compared with that of older children with this disease subtype, which possesses unique clinical and biologic features. The major problem in treatment of infant ALL is the occurrence of early relapses, justifying early intensive chemotherapy. The role of BMT in infants is debatable. Large collaborative studies are the only way to ensure further improvement of therapy for most infants with ALL.

The international Interfant-99 group is analyzing the efficacy of a so-called hybrid regimen, in which especially low- and high-dose ara-C is added to an "ALL-based regimen", that includes high-dose MTX and dexamethasone but does not contain drugs imposing potential long-term risks. The combined CCG/POG study groups are testing the efficacy and toxicity of intensified chemotherapy with allogenic BMT in infant ALL. These studies might lead to stepwise increases in the cure rate, similar to the progress achieved in the last 10-15 yr for infants with ALL. Also, they will undoubtedly resolve which risk factors have the strongest independent prognostic value and thus point the way to more specific therapies for subgroups of infant ALL patients.

Molecular studies, especially those focusing on *MLL* gene, drug resistance, and minimal residual disease might contribute further to the development of specific therapies. Pharmacokinetic studies are needed to improve drug dosing in infants and to prevent undertreatment owing to unnecessary dose reductions. New innovative approaches, such as drugs that target the products of chimeric oncogens, are probably needed to increase the cure rate to the same rate as that in older children with ALL.

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4 Biology and Treatment of Acute Leukemias in Infants

Perspective 2

GREGORY H. REAMAN

CONTENTS

1. INTRODUCTION

Acute leukemia occurring during the first year of life is characterized by unique epidemiologic, clinical, and biologic characteristics and exhibits gender-specific differences in incidence and distribution frequencies distinct from those of acute leukemia during childhood *(1–4)*. Whereas the incidence of acute lymphoblastic leukemia (ALL) is nearly four times that of acute myeloid leukemia (AML) in children older than 1 yr, the incidence of ALL is only twice that of AML in infants *(5)*. Infants account for approx 3% of ALL cases diagnosed annually in the United States, but they represent 10–12% of cases of AML diagnosed each year.

Infants with ALL, as well as AML, present with a somewhat unique constellation of features, including hyperleukocytosis, massive organomegaly, and central nervous system (CNS) involvement at diagnosis *(1,2)*. Biologically, the leukemic lymphoblasts of infants display an immunophenotypic pattern that differs significantly from those generally observed in older children *(3,6,7)*. There is also a high incidence of specific nonrandom structural chromosomal abnormalities, notably t(4;11), in most infants with ALL, and molecular rearrangements of the *MLL* gene within chromosome band 11q23, the site of the t(4;11) breakpoint, are observed in a significant number of infants with either ALL or AML *(1,4,8–11)*.

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2. EPIDEMIOLOGY AND BIOLOGIC CLUES TO ETIOLOGY

The incidence of acute leukemia in the first year of life in the United States is approx 30 cases per million live births and is increasing annually. The annual incidence of ALL is 20 per million infants at risk and exceeds that of AML, which is 10.6 per million infants under 1 yr of age *(5,12,13)*. The peak incidence of AML in childhood occurs during the first year of life, in contrast to the peak age incidence of ALL, which occurs between the ages of 2 and 4 *(5,12)*. Distinct gender differences in the incidence of ALL are observed in infants. Among older children, the disease occurs more often in boys, whereas the reverse is true in infancy *(2,13,14)*. A slight excess of AML in infant girls is also seen, contrasted with the lack of a gender-associated difference in older children *(5)*.

Cytogenetically or molecularly assessed gene rearrangements, including specific rearrangements or translocations of the *MLL* (*ALL1*, *HRX*, and *HTRX-1*) gene on chromosome band 11q23, occur in nearly 80% of infants with ALL *(4,8,10,12,15,16)* and in up to 50% of infants with AML *(8,17,18)*. Approximately 80% of the infants with monoblastic (M4, M5) variants of AML exhibit *MLL* rearrangements *(18,19)*. Up to 10% of AML cases in infancy are associated with myelodysplastic syndromes, notably monosomy 7 and del (7q) syndromes, and with Down's and Noonan's syndromes as well as neurofibromatosis type 1, whereas ALL in infants does not appear to be associated with specific constitutional syndromes,

although clear associations exist in children diagnosed at older ages *(20–23)*.

Infants have been the focus of several epidemiologic investigations of acute leukemia. Since environmental and/or occupational exposures to ionizing radiation, petroleum solvents, and pesticides are correlated with an increased risk of AML in adults, early epidemiologic studies concentrated on such associations *(4,24,25)*. Early case control studies of the Children's Cancer Group (CCG) demonstrated that parental pesticide exposure and maternal use of marijuana and ethanol during pregnancy were significantly associated with AML in infants and younger children (younger than 3 yr of age) and particularly associated with the FAB M4 and M5 subtypes *(25,26)*. Adverse maternal reproductive history with history of previous fetal loss has also been associated with an increased incidence of AML in infants *(27–29)*.

Particular focus on the molecular epidemiology of infant leukemia results from the frequent association of both ALL and AML with rearrangements of the *MLL* gene in up to 80 and 50% of infants, respectively. Identical abnormalities of the *MLL* gene at band 11q23 are involved in these rearrangements *(30)*, which may involve a variety of partner chromosomes, including 4, 9, and 19.

Rearrangements of the *MLL* gene have also been observed in treatment-related AML, specifically cases associated with DNA topoisomerase II inhibitors, such as the epipodophyllotoxins, etoposide, and teniposide *(31,32)*. The strong association between exposures to topoisomerase II inhibitors and the development of acute leukemia with *MLL* rearrangements has led to an intriguing hypothesis that maternal exposure to naturally occurring topoisomerase II inhibitors during pregnancy could increase the risk of infant leukemia *(33,34)*. A number of natural and synthetic topoisomerase II inhibitors exist, including flavonoids (quercetin, present in fresh fruits and vegetables, and genistein), catechins (present in cocoa), caffeine, quinolones (present in frequently used antibiotics), thiram (an agricultural fungicide), certain benzene derivatives, and Chinese herbal medicines *(34)*. Podophyllin, used to treat genital warts, is also a topoisomerase II inhibitor *(35)*. Preliminary data strongly suggest that maternal exposure to topoisomerase II inhibitors, particularly in the diet, is positively associated with AML in infants *(36,37)*. Soy-based formulas may be associated with exposure to high levels of isoflavones, including daidzein and genistein. Because human feeding studies demonstrate that the bioavailability of these compounds may be much higher than previously believed *(38)*, they might also have a role in leukemia induction through the inhibition of topoisomerase II *(38)*.

Very briefly, the topoisomerase II-active agents stabilize the topoisomerase II/DNA covalent complex, leading to the persistence of DNA double-strand breaks. There is in vitro evidence, in both malignant and normal cells, that the *MLL* gene is a target for topoisomerase II inhibition *(32,39)*. Understanding the role of the *MLL* gene in the etiology of leukemia in infants has been significantly advanced by investigations of monozygotic infant twins *(40,41)*. Although the incidence of acute leukemia in infant twins is rare, detailed cytogenetic and/or molecular studies have demonstrated unique and identical, clonal molecular *MLL* gene rearrangements in cases of ALL with t(4;11) and t(11;19), as well as AML with t(9;11) and t(11;22) *(42–44)*. The nonconstitutional origin of these rearrangements suggests that the translocations develop *in utero*. The concept that *MLL* rearrangements in twins develop as *in utero* events justifies continued efforts to evaluate prenatal exposure to environmental agents, such as dietary DNA topoisomerase II inhibitors. Other evidence for prenatal events resulting in infant leukemogenesis is provided by Gale et al. *(45)*, who found a leukemia-specific chromosomal translocation, t(4;11), at the time of birth in infants whose leukemia was not diagnosed until 5 and 24 mo later. These results were based on polymerase chain reaction amplification of genomic DNA obtained from routine neonatal blood spots.

The association of *MLL* gene rearrangement and translocations with the pathogenesis of leukemia has been extended by knockout mouse models demonstrating defective yolk sac hematopoiesis *(46,47)* and further suggested by the observation that introduction of t(9;11) in these knockout mouse models results in the development of leukemia following a latency period, implicating a pivotal role for the *MLL* gene in leukemogenesis *(48)*.

Sixty percent of *MLL* gene rearrangements, including translocations, are identified by standard cytogenetic techniques, the remainder being detected by molecular analysis or fluorescence *in situ* hybridization (FISH) studies with *MLL*-specific probes *(18)*. The translocation breakpoints in leukemias in infants, as well as in treatment-related leukemias caused by topoisomerase II inhibitors, involve identical breakpoint cluster regions, an 8.5-kb region between exons 5 and 11 *(4,49,51)*. *MLL* gene translocations involve about 30 different partner genes, encoding protein products of multiple types *(52)*. The *AF4* gene at chromosome 4q21, a transcriptional, *trans*-activating gene, appears to be the most common partner associated with *MLL* rearrangements in ALL, whereas the *AF9* gene at chromosome 9p22 and the *ENL* gene at chromosome 19p13 are often common partner genes in AML *(11,53–58)*. The proteins encoded by most 11q23 partner genes appear to bear little or no homology and may encode different types of functional elements with resultant chimeric proteins operating in distinct ways in leukemogenesis *(59,60)*. Since *MLL* may interact with DNA or other DNA binding proteins, the various chimeric proteins may activate or repress distinct genes, resulting in diverse leukemias, possibly with different responses to treatment and, therefore, dissimilar outcomes.

A remaining unresolved question regarding the molecular etiology of infant leukemia, specifically ALL with t(4;11), is whether expression of the *MLL-AF4* fusion gene is sufficient to lead to the fully transformed phenotype *(61,62)*. The latency period to leukemia onset in the murine knockout mouse model and observations of human latency suggest that leukemogenesis requires genetic changes in addition to *MLL* translocations *(48)*. Detection of *MLL* partial duplications in the peripheral blood and marrow in normal adult subjects and the finding of *MLL-AF4* fusions in normal fetal liver and normal infant marrow also suggest that these leukemia-associated translocations alone may be insufficient as etiologic events *(41,42,45,63)*. Thus, expression of genes other than *MLL* and *AF4* could be

involved in leukemogenesis in infants. Mutations of *p53*, *Ras*, and *p16* are not consistent alterations in this age-related subtype of acute leukemia.

A recent finding of homozygous deletions of exons of the *Ikaros* gene with expression of dominant-negative *Ikaros* isoforms in six of seven infants who exhibited *MLL-AF4* fusion suggests that disruption of normal *Ikaros* function may contribute to leukemogenesis associated with t(4;11) *(64)*. Inappropriate expression of non-DNA binding *Ikaros* isoforms during early lymphopoiesis may dysregulate normal lymphocyte development, leading to maturational arrest at discrete stages of lymphocyte ontogeny, predisposing lymphocyte precursors to "second hits" and leukemic transformation. Previous studies in mice have demonstrated that germ-line mutant mice expressing dominant-negative isoforms of *Ikaros* also develop ALL *(65,66)*. An abundance of dominant-negative mutant Ikaros isoforms that no longer bind DNA could interfere with centromeric recruitment and expression of specific genes during lymphocyte development. A lack of lineage-specific gene silencing could also explain the "lineage infidelity" demonstrated by myeloid antigen expression in ALL in infancy *(67)*. Obviously, further investigations of other molecular genetic abnormalities will be important in clarifying the leukemogenic role of such rearrangements and may provide clinically exploitable information for developmental therapeutics as well as disease prevention.

3. TREATMENT RESULTS

Age-associated treatment results are clearly evident in childhood ALL, with infants and older adolescents/young adults having significantly worse outcomes. Similar age-related differences in event-free survival have not been observed in AML. Although early reports suggested a high degree of success with epipodophyllotoxin-based chemotherapy for infants with AML, particularly those with the M4 and M5 subtypes *(68)*, subsequent trials have not confirmed these findings. In a CCG study of 17 infants with monoblastic leukemia, therapy with etoposide yielded satisfactory induction rates without any appreciable impact on the long-term, event-free survival rates achieved with conventional intensive multiagent chemotherapy *(69,70)*. In recent AML studies, the FAB M4 and M5 morphologic subtypes and 11q23 breakpoint abnormalities did not appear to be adverse prognostic features in infants with AML. Treatment with intensively timed induction chemotherapy, followed by HLA-matched, related-donor bone marrow transplantation, improved event-free survival in children and in younger adults with AML, and age-specific differences were not apparent *(71)*.

In a recent report of 40 infants younger than 2 yr who were undergoing allogeneic bone marrow transplantation for AML, following preparative regimens of busulfan and cyclophosphamide or cyclophosphamide and total-body irradiation (TBI), the neurologic development in survivors was appropriate for age; however, TBI had deleterious effects on growth *(72)*. The observation that t(9;11) is a favorable prognostic factor in infants with AML has resulted in controversy as to whether bone marrow transplantation at first remission is even necessary for this subgroup of patients. Otherwise, there appears to be no clinical justification for the development of unique treatment strategies for infants with AML. Results of a Medical Research Council (United Kingdom) trial, which combined cytarabine and daunorubicin with either etoposide or thioguanine as induction therapy followed by postinduction consolidation therapy that included matched sibling stem cell transplantation, indicated superior disease-free survival and a lower relapse rate in infants compared with older children, but overall survival rates were identical *(71)*.

As cure rates in children with ALL approach (and in some subgroups of patients exceed) 80%, the recognition of specific populations of children with unfavorable responses to treatment has become even more apparent. Although comprising only 3% of childhood ALL cases, infants pose the most formidable challenge to therapists. Despite major advances in cure rates for the general patient population, achieved through the identification of prognostic factors and the implementation of risk-adjusted therapy, the long-term event-free survival of infants with ALL approximates 40% *(73–78)*. This result, although only half that being achieved in standard-risk ALL patients, or in high-risk patients treated with intensified therapy regimens, represents an improvement over the historical experience. This modest success was accomplished through clinical trials and efforts to explore the biologic differences between leukemias in infants and older children. Retrospective reports of several series of infants with ALL, the largest dealing with infants treated on a number of consecutive clinical trials of the CCG, revealed 3-yr, event-free survival rates of only 20%. Early treatment failure, characterized by both systemic and extramedullary relapse, rather than therapy-related toxicity, explained this poor outcome *(2)*.

A number of clinical features, including hyperleukocytosis, splenomegaly, CNS leukemia at diagnosis, and a poor early response to induction chemotherapy, have been universally reported in infants with ALL *(2,3,79,80)*. Specific biologic features, including the lack of CD10 expression and immunophenotypic coexpression of myeloid-associated antigens, are also frequently seen *(3,74)*.

The improvement in treatment outcome in infants with ALL, although decidedly less impressive than improvements seen in older children, have been accomplished through progressive intensification of systemic chemotherapy. Table 1 details representative treatment results obtained by several groups over the past 5 yr. The largest series of similarly treated patients, representing the results of two consecutive single-arm studies of the CCG, incorporated intensive four-drug induction therapy and postinduction consolidation and intensification with cytarabine and cyclophosphamide. One of the most significant accomplishments was the prevention of CNS relapse with the use of high-dose systemic as well as intrathecal methotrexate and cytarabine, eliminating the need for cranial irradiation and resulting in a cumulative risk for CNS relapse of only 3%, despite a 14.2% prevalence of CNS leukemia at diagnosis *(2)*. Intensification of therapy for infants has also included the combination of mitoxantrone and cytarabine *(77,81)*, intermediate-dose methotrexate infusions *(82)*, and combinations of cytarabine and etoposide *(76,83,84)*. In Medical Research Council (United Kingdom) ALL trials, short-term, highly intensive chemotherapy, followed by autologous or allogeneic

Table 1
Recently Reported Treatment Results for ALL in Infants

Study	*Year Reported*	*No. of Patients*	*Outcome*	*Treatment advance*	*Ref.*
POG 8493	1997	82	4-yr EFS, 28 ± 5%	No significant improvement	*79*
POG	1998	33	5-yr EFS, 17 ± 8%	Postinduction intensification with alternating drug pairs	*82*
EORTC-CLCG	1994	28	4-yr EFS, 43 ± 19%	Cyclo/ara-C consolidation; HD (2.5 g/m^2) MTX; sequelae of cranial irradiation	*78*
MRC-UKALL VIII/X	1994	88	5-yr EFS, 40% (>6 mo) and 40% (<6 mo)	ara-C/etoposide consolidation; HD ara-C/mitoxantrone reinduction allo/auto BMT	*77*
DFCIC	1997	23	4-yr EFS, 54% ± 11%	Intensive anthracycline/L-A/sp; many late sequelae of therapy and cranial irradiation	*76*
CCG					
107	1999	99	4-yr EFS, 33 ± 4.7	HD ara-C/Cyclo consolidation	*75*
1883		135	4-yr EFS, 39 ± 4.2%	HD MTX/IT MTX/ara-C; decreased CNS relapse rate; normal developmental outcomes	
BFM					
83	1998	105[a]	5-yr EFS, 23% ± 12%	Delayed intensification[a]	*81*
86			5-yr EFS, 37% ± 8%	Prednisone response: 5-yr EFS, 53 ± 6	
90			5-yr EFS, 51% ± 7%	vs 14% ± 7% for good vs poor responders[b]	

Abbreviations: POG, Pediatric Oncology Group; EORTC-CLCG, European Organization for the Research and Treatment of Cancer-Children's Leukemia Cooperative Group; MRC-UKALL, Medical Research Council, United Kingdom, ALL trials; DFCIC, Dana-Farber Cancer Institute Consortium; CCG, Children's Cancer Group; BFM, Berlin-Frankfurt-Münster Group; EFS, event-free survival; Cyclo, cyclophosphamide; HD, high dose; IT, intrathecal; ara-C, cytarabine; MTX, methotrexate; allo/auto BMT, allogeneic/antologous bone marrow transplantation; L-Asp, L-asparaginase.

[a]Includes all three BFM studies.

[b]BFM-86/90.

stem cell transplantation, did not result in improved event-free survival, owing to therapy related toxicity and deaths in remission *(77)*. Significant long-term toxicity has also emerged as a consequence of other intensive chemotherapy regimens based on intensive anthracycline and L-asparaginase in a series from the Dana-Farber Cancer Institute Consortium *(76)*. Although therapy intensification improved event-free survival in a small series of infants, developmental delays, learning disabilities, asymptomatic cataracts, asymptomatic echocardiographic abnormalities, and short stature were experienced by 82%, 67%, 30%, and 18% of 13 long-term survivors, respectively.

Attempts to improve clinical outcome through intensification of therapy will require systematic evaluation of specific agents, given the unique biologic characteristics and the unique drug resistance profile of leukemic lymphoblasts in infants. Using an in vitro drug resistance assay (MTT assay), Pieters et al. *(85,86)* reported an association between in vitro resistance to glucocorticoids and L-asparaginase and poor prognosis of ALL in infants compared with older children. They also reported an association between resistance profiles and lack of CD10 expression *(85,86)*. This same study also demonstrated that ALL cells from infants exhibit enhanced sensitivity to cytarabine. Determination of the optimal dose of cytarabine, particularly in the high-dose setting, may provide additional opportunities to intensify therapy and improve outcome. In fact, the use of high-dose cytarabine may partly explain the differences in event-free survival between the two consecutively reported trials of the CCG *(75)*. A portion of the therapy intensification employed by investigators of the Dana-Farber Cancer Institute Consortium included high-dose cytarabine as well *(76)*. In addition, successful treatment of relapsed ALL in a small number of infants has been reported with the use of an intensive antimetabolite-based salvage regimen, which included high-dose cytarabine throughout therapy and may have contributed to the cure of these infants *(87)*. In vitro drug resistance profiles demonstrate that the leukemic lymphoblasts from infants with ALL exhibit a high sensitivity to cytarabine. Evaluation of cytarabine sensitivity with the MTT assay demonstrated a 2.4-fold increase in sensitivity in leukemic cells from infants younger than 1 yr of age compared with that in leukemic blasts isolated from the pretreatment marrow of children between 1 and 10 yr of age *(86)*. Leukemic cells with an early B-cell precursor phenotype lacking CD10 expression also had a more than twofold-increased sensitivity to cytarabine.

Other evidence for the use of this agent is the impressive long-term event-free survival rate, approaching 50%, in t(4;11)-positive adult ALL treated on two German multicenter trials with a

regimen containing high-dose cytarabine and mitoxantrone *(88)*. The optimal use of this agent requires careful evaluation, since there is generally a paucity of pharmacokinetic data related to antileukemic drugs in infants. In the CCG studies, cytarabine dosage was arbitrarily reduced in young infants because of concern over increased toxicity. However, limited data suggest that the clearance of this agent and its systemic exposure may be similar in infants and older children, suggesting that empiric reduction of cytarabine dosage may not be necessary. Hence, further dose intensification may advance improvement in clinical outcome. The optimal treatment strategy for ALL in infants requires further coordinated and collaborative investigation.

4. LATE EFFECTS

A major consideration in the treatment of ALL in infants is the considerable potential for toxicity, both acute and long-term, associated with successful antileukemic therapy. Although the development and physiologic maturity of many major organ systems may not be maximal in infants by the time of diagnosis, the developing CNS has special relevance, given its unique sensitivity to toxic insult and potential for disastrous long-term complications *(89)*.

Since infants with ALL constitute the group of patients with the highest incidence of CNS disease and extramedullary relapse involving the CNS, adverse sequelae related to conventional CNS-directed therapy incorporating cranial radiation is of great concern. In a historical series, the small number of infants who survived ALL after receiving cranial radiation as part of CNS preventive therapy exhibited debilitating neuropsychological sequelae *(2)*. Two additional recent studies reported high rates of developmental delay and significant learning disabilities related to neurotoxicity attributed to cranial radiation *(76,89)*. Thus, therapeutic strategies specifically designed for infants to mitigate the disastrous neuropsychological sequelae associated with cranial radiation have included more frequent use of intrathecal chemotherapy *(79,82)* and the introduction of very-high-dose, protracted systemic infusions of methotrexate *(2,90)* as an alternative to CNS-directed therapy. Substantial reductions in the rate of isolated CNS relapse in two consecutive studies of the CCG have been observed in patients receiving CNS-directed therapy consisting of protracted (24-hr), very-high-dose (33.6 g/m^2) methotrexate infusions with leucovorin rescue plus intensive intrathecal therapy with both methotrexate and cytarabine. Compared with historical controls, wherein the CNS relapse rates exceeded 20%, relapse rates of 9 and 3% in each of these two consecutive trials represented significant treatment advances *(2)*.

Developmental and neuropsychological evaluations of long-term survivors from the earlier of these two studies have demonstrated mean scores on standardized cognitive and motor tests in the average range, with a normal distribution of scores by comparison with population-based standards *(91,92)*. These findings suggest a positive early developmental outcome for these children and represent a substantial improvement over the neurocognitive potential of previously treated infants.

To date, no other long-term clinical complications in patients, now followed for up to 8 yr after successful completion of therapy, have emerged. Effective strategies to prevent CNS relapse while eliminating the potential for adverse neurocog-nitive and neurodevelopmental sequelae are crucial to current and future clinical trials investigating the optimal management of ALL in infants.

5. PROGNOSTIC FACTORS

The prognosis for infants with ALL depends on the presence or absence of certain presenting clinical features, including age younger than 6 mo, hyperleukocytosis, lack of CD10 expression, myeloid-associated antigen expression, and 11q23 breakpoint abnormalities (*MLL* rearrangements) *(1–3,6,9,74,75,93,94)*. Response to therapy is also important in predicting the ultimate event-free survival rate in infants with ALL. The initial response to therapy has emerged as a significant prognostic indicator in childhood ALL, whether assessed by the degree of leukemic cytoreduction in the bone marrow by d 7 or 14 of induction therapy or by the reduction in peripheral blood lymphoblasts owing to a 7-d prophase course of prednisone and intrathecal methotrexate *(95,96)*. Response to prednisone has emerged as the strongest prognostic factor in infant ALL in studies of the Berlin-Frankfurt-Münster Group *(81)*. Response to prednisone predicted a superior event-free survival rate (53% vs 14.7%) and will be used as the only stratification for a European intergroup effort (INTERFANT) to improve the treatment of ALL in infants. Recently reported CCG experience demonstrated a threefold excess risk of treatment failure in infants whose bone marrows had not attained an M1 status by d 14 of therapy. The markedly predictive significance of early response to therapy persisted after correction for age, leukocyte count, and *MLL* rearrangements *(75)*.

In all studies evaluating prognostic factors in infants, patient numbers are generally small, and there are significant interrelationships among such prognostic variables as *MLL* rearrangements, lack of CD10 expression, hyperleukocytosis, and age younger than 6 months. Thus, continued evaluation to ascertain the independent nature of prognostic variables is needed *(1,67,74,97,98)*. Multivariate analysis using a Cox regression model to examine event-free survival in a group of 94 infants with complete data for the variables tested demonstrated independent prognostic significance for the following factors: M1 bone marrow on d 14 of induction therapy, age 6 mo or older, leukocyte count $\geq 50 \times 10^9$/L, CD10 expression, and absence of t(4;11) by cytogenetic evaluation *(75)*.

Considerable controversy exists regarding the prognostic significance of *MLL* rearrangements. In trials reported by the CCG, only t(4;11), associated with *MLL-AF4* fusion, was associated with a dismal outcome; other 11q23 breakpoint abnormalities were not prognostically significant *(10,11)*. However, conclusions regarding the prognostic significance of *MLL* rearrangement and the importance of specific partner genes and their association with outcome require larger series of patients and uniform laboratory assessments, i.e., cytogenetics or more sensitive molecular determinations or FISH *(1,9)*. At present, multiple bodies of evidence confirm the dire prognosis of infants younger than 6 mo with t(4;11) *(11,83,94,98)*. In a recent CCG study, infants with no 11q23 abnormalities or 11q23 abnormalities other than t(4;11) had

5-yr event-free survival rates of 57.1 and 46.4%, respectively, compared with only 4.8% for infants whose blasts did harbor t(4;11) *(11)*.

6. FUTURE PERSPECTIVES: BIOLOGIC AND THERAPEUTIC INVESTIGATIONS

Given the clinically unique characteristics of acute leukemia in infants and the similarity of some clinical features in cases of infant ALL and AML with identical gene rearrangements, further molecular epidemiologic studies specifically on exposure to DNA topoisomerase II inhibitors, as well as additional gene rearrangements that may be required for leukemogenesis in infants, and the timing of such events, will prove invaluable in furthering our understanding of the etiology and pathogenesis of this disease. Although the clinical and molecular similarities between ALL and AML, particularly those with *MLL* gene rearrangements, suggest to some investigators that the optimal therapy for both diseases might be the same, such a combined approach for acute leukemia in infants has not yet been tested. Furthermore, there is no agreement on the combined therapeutic approach. The use of intensively timed induction therapy and allogeneic stem cell transplantation may not be justified for infants older than 6 mo who lack t(4;11) or other *MLL* rearrangements *(69,70,99)*.

Rather than a uniform therapy for ALL and AML in infants, improved treatment results with intensification of therapy, successful induction rates with conventional ALL induction, and the suggestion that improvement in outcome may be attributable to the use of high-dose cytarabine provide justification for the development of a "hybrid" regimen incorporating agents such as anthracyclines, cytarabine, and etoposide with vincristine, prednisone, L-asparaginase, and antimetabolites. High-dose cytarabine consolidation following standard intensive four-drug induction plus cyclophosphamide and etoposide is being evaluated in parallel single-arm pilot studies of the CCG and the Pediatric Oncology Group. Following the intensified induction therapy, patients receive consolidation therapy incorporating very-high-dose (33.6 g/m^2), protracted (24-hr) systemic MTX infusions and intrathecal chemotherapy followed by a 6-mo intensified maintenance preceding a standard antimetabolite-based maintenance regimen. This strategy has resulted in projected 3-yr event-free survival rates exceeding 65% (P. Dinndorf and Z. Dryer, personal communication).

Resolving the controversy regarding the prognostic significance of *MLL* rearrangement and the specific contribution of *MLL-AF4* fusion to an adverse outcome is necessary before adopting related therapeutic strategies in infants with ALL and AML with t(4;11). A trial of the Medical Research Council of the United Kingdom concluded that excessive treatment-related morbidity and mortality and a high rate of relapse among a relatively small series of infants prospectively treated with matched sibling donor transplants in first remission did not justify this approach *(77)*. Although the trials were not controlled, the responses of infants treated on recently reported CCG trials of infants transplanted in first remission were similarly disappointing *(75)*.

Bone marrow transplantation in first remission for ALL as well as AML in infants has significant theoretical and practical concerns. The major practical limitation is that few infants have HLA-matched sibling donors, necessitating the use of alternative donor sources, which may be associated with greater risks *(100)*. In AML trials of the Medical Research Council, United Kingdom, transplantation in first remission was not superior to intensive consolidation chemotherapy, in contrast to the U.S. experience *(70,100,101)*. Reports of small series of patients indicate that allogeneic matched sibling-donor transplants are feasible and successful and are associated with a paucity of adverse long-term sequelae *(102–104)*. A recent series of seven infants with ALL transplanted with matched sibling-donor marrow or umbilical cord blood following pretransplant conditioning (which included TBI, etoposide, and cyclophosphamide) demonstrated no excessive transplant-related toxicity and successful engraftment in all patients within a median of 18 d. Four of the seven patients are surviving without evidence of leukemia with a median follow-up of 2 yr *(105)*. Additional support for therapy strategies incorporating allogeneic stem cell transplantation in infants is provided by experience at the Fred Hutchinson Cancer Center. Seven of nine infants transplanted in first remission at various times from diagnosis continue in complete remission for periods ranging from 2 to 11 yr post transplant without inordinate long-term toxicity (J. Sanders, personal communication). Further follow-up of other small patient groups will hopefully provide useful information about extending the applicability of stem cell transplantation as a potential therapeutic innovation in infants with ALL. The proof of principle will require a controlled clinical trial.

Other novel therapeutic initiatives include the use of specific anti-CD19 immunotoxins, which have demonstrated in vivo efficacy in a severe combined immunodeficiency (SCID) mouse model of B-cell precursor ALL with 11q23 rearrangements *(106)*. A small number of infants have been safely and successfully treated with B43-PAP, an anti-CD19 murine monoclonal antibody linked to pokeweed antiviral protein *(107)*. Evaluation of other antibody toxin conjugates may be evaluated.

The recently described SCID mouse model of human infant ALL with molecular evidence of *MLL* rearrangement and evidence of *MLL-AF4* fusion should prove useful in evaluating and screening both cytotoxic and biologic agents for efficacy and their potential use in this high-risk group of patients. This methodology may prove valuable in elucidating in vivo mechanisms of drug resistance *(108)*.

The efficacy of intensive therapy approaches incorporating cytarabine with synergistic drug combinations requires further investigation. In vivo screening of potential new agents with preclinical models, as well as assessing their in vitro efficacy in the MTT assay, may prove helpful in prioritizing which new agents or classes of agents should be pursued developmentally *(109–111)*. Furthermore, pharmacokinetic investigation of existing active agents as well as new agents are needed in planning optimal dosing and scheduling strategies based on physiologic parameters, precluding the need for arbitrary dose modifications.

Despite progressive improvements in event-free survival, infants with ALL remain the group at highest risk for treatment failure, despite increasingly intensive multiagent chemo-

therapy regimens. Further investigation of clinical and biologic characteristics may prove useful in refining the risk stratification of patients for whom novel therapeutic strategies, including alternative stem cell transplantation and targeted immunotherapy, may be indicated. Therapeutic investigations in acute leukemia in infants should explore unique biologic targets in this group of patients, notably functional properties of *MLL* rearrangements and fusion products dependent on partner gene translocations

Infants with ALL represent only 3% of children with this disease, and in North America <60 infants a year accrue to trials of the two pediatric cooperative groups that collectively capture nearly all the pediatric leukemia cases seen. In light of the unique biologic characteristics of ALL in infants, the focus on developing treatment approaches aimed at minimizing acute and long-term toxicities, and the relative paucity of cases, justification exists for international cooperation and collaboration in evaluating novel therapeutic interventions and testing them in controlled trials in this group of challenging patients.

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CHEMOTHERAPEUTIC STRATEGIES

II

CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

B

5 Treatment of Childhood Acute Lymphoblastic Leukemia

Martin Schrappe and Martin Stanulla

CONTENTS

1. INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. It accounts for approx 25% of all childhood cancers and almost 75% of childhood leukemias *(1,2)*. Treatment results in childhood ALL are one of the true success stories of modern clinical oncology, with an overall cure rate of 65% to almost 80% by application of intensive multiagent chemotherapeutic regimens *(1–17)*. However, only a few study groups worldwide have presented data on unselected patient populations, thus limiting outcome comparisons of different treatment protocols. Most published reports address specific patient subsets only. In our most recently completed therapy study within the Berlin–Frankfurt–Münster (BFM) study group, ALL-BFM 90, the overall probability of event-free survival at 6 yr for all 2178 patients (≤18 yr) was 78% (standard error, 1%) *(15)*. By comparison, in the recent British UKALL-X study *(5)*, a 5-yr disease-free survival of 62% was achieved in 1612 patients (0–14 yr). Patients randomized to receive two intensification treatments fared best in that trial, with a 5-yr disease-free survival of 71%. Investigators from the Dana-Farber Cancer Institute (DFCI) reported excellent treatment results in two subsequent trials that included patients of the same age range as in the ALL-BFM 90 trial: their event-free survival was 72% in trial 81-01 and 78% for 220 patients in trial 85-01 *(11,14)*. The last two treatment programs of the St. Jude Children's Research Hospital resulted in 4-yr probability of event-free survival of 73% ($n = 358$; 0–18 yr) in study XI and in a 5-yr-event-free survival of 67% ($n = 188$) in study XII *(7,12)*. In the latter trial, patients with B-lineage ALL who received pharmacologically adapted doses of methotrexate, cytarabine, and VP-16 (etoposide) achieved a 5-yr event-free survival of 76%, compared with 66% for patients with standard medication.

In this chapter, we try to explain some of the important principles of treatment of childhood ALL and summarize some of the important steps that have been made to arrive at these concepts. Apologies are made to those whose research may have been omitted. Also, this chapter excludes mature B-cell ALL or French–American–British (FAB) classification L3, as it is now successfully treated by a non-Hodgkin's lymphoma (NHL) therapy-based approach *(18–20)*.

2. DIAGNOSTIC ISSUES

The clinical presentation of a child with ALL largely depends on the extent of the leukemic infiltration of the bone marrow and extramedullary sites. Typical clinical signs are fever; pallor; fatigue; bruises; enlargement of liver, spleen, and lymph nodes; and pain (e.g., bone pain). In most patients, white blood cell (WBC) counts show anemia, thrombocytopenia and granulocytopenia, with or without concomitant leukocytosis. In any case, the diagnosis of ALL has to be confirmed by bone marrow (BM) aspiration and in particular cases by Jamshidi needle biopsy.

2.1. Morphology

The diagnosis of ALL is established when at least 25% lymphoblasts are present in the BM, or when blasts are present in the peripheral blood (PB) or the cerebrospinal fluid (CSF). BM and blood smears as well as CSF cytospin preparations are usually stained using a modified Wright staining technique and cytochemistry reactions (periodic acid-Schiff reaction, acid phosphatase, α-naphthyl acetate esterase, and myeloperoxidase reaction) and evaluated according to FAB criteria *(21)*.

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Table 1
Distribution of Standard- and High-Risk Childhood ALL Cases According to NCI Criteria Based on Data from Four ALL-BFM Trials

Risk group	*Definition*	*% of B-lineage ALL*
Standard	WBC at diagnosis < 50×10^9/L and age at diagnosis 1–9 yr	~ 75
High	WBC at diagnosis ≥ 50×10^9/L or age at diagnosis ≥ 10 yr	~ 25

Abbreviations: NCI, National Cancer Institute; WBC, white blood cells.

2.2. Central Nervous System Disease

Central nervous system involvement, stage 3 (CNS3), is diagnosed if >5 cells/μL are counted in a non-blood-contaminated CSF sample and if lymphoblasts are identified unequivocally, or if intracerebral infiltrates are detected on cranial computed tomography *(22)*. CNS2 refers to an intermediate state in which <5 cells/μL CSF are detected, but blasts are unequivocally indentified. CNS1 describes negativity for CNS disease (<5 cells/μL CSF, no blasts). When diagnostic issues arise, CSF samples should also be analyzed after cytospin preparation, a method through which cellular components within the CSF are concentrated by centrifugation.

2.3. Immunophenotype

Immunophenotyping procedures are extensively described elsewhere *(23,24)* and by Behm, Ludwig, and colleagues in Chapters 1 and 2. Briefly, surface antigens are considered positive if ≥20% of the leukemic cells express the antigen with >98% fluorescence intensity, compared with negative control cells. Positivity for terminal deoxynucleotide transferase (TdT) and cytoplasmic (cy) antigens is defined as >10% of the cells exhibiting nuclear (TdT) or intracytoplasmatic (cyIgM, cyCD3) fluorescence. In the early 1990s, two-color flow cytometric analysis with appropriate monoclonal antibodies directly conjugated to fluorescein isothiocyanate or phycoerythrin was introduced. In BFM trials, immunophenotypic subgroups are defined according to the European Group for the Immunological Characterisation of Leukaemias (EGIL), as follows: *Pro-B ALL*: TdT+, CD19+, CD10–, cyIgM–, surface immunoglobulin (sIg)–; *common (c) ALL*: TdT+, CD19+, CD10+, cyIgM–, sIg–; *pre-B-ALL*: TdT+, CD19+, CD10+/–, cyIgM+, sIg–; and T-ALL: TdT+, cyCD3+, CD7+ *(25)*. Coexpression of myeloid antigen(s) is defined as simultaneous expression of one or more of the myeloid lineage-associated molecules tested (CD13, CD33, and CD65s) on at least 20% of the lymphoblasts.

2.4. DNA Index

Cellular DNA content is determined by flow cytometry, as described elsewhere *(26)*. The DNA index of leukemic blasts is defined as the ratio of DNA content in leukemic G0/G1 cells to that of normal diploid lymphocytes. In this assay, a widely accepted cutoff point at 1.16, which correlates with >50 chromosomes/cell, is used to distinguish prognostic categories.

2.5. Cytogenetic and Molecular Genetic Analysis

Standard cytogenetic techniques are described elsewhere *(27)*. During the 1990s, deeper insight into chromosomal translocations at the molecular level allowed the development and evaluation of reverse transcription (RT)-polymerase chain reaction (PCR)-based screening for the *BCR/ABL*, different *MLL*, and *TEL/AML1* fusion products in most of the large study groups on childhood ALL *(3,28)*. Thus, today, a combined approach using cytogenetic and molecular genetic techniques is often employed at initial diagnosis. These techniques, as well as flow cytometry, are also used to monitor disease burden during therapy by detecting, for example, leukemia clone-specific characteristics such as immunoglobulin or T-cell receptor gene rearrangements *(29)*.

3. RISK ASSESSMENT

Adjustment of therapy according to the risk of treatment failure (standard/low, intermediate, or high) has become a common feature of the clinical management of childhood ALL. Thus, the identification of prognostic factors has become an essential element in the design, conduct, and analysis of clinical trials in childhood leukemia over the last two decades. These factors mostly include clinical and biologic characteristics that are assessable at diagnosis *(1–3)*. In addition, several study groups evaluated a variety of estimates of early response to treatment as a prognostic factor for treatment allocation *(9,15,30–37)*. The issue of response to treatment has become controversial, as discussed in more detail below. It is well recognized that the significance of prognostic factors cannot easily be channeled into a uniform statement for all study groups, since virtually all these factors are associated with the type and the intensity of treatment administered. Furthermore, additional differences between study groups (e.g., eligibility criteria and ethnic or racial composition of study populations) have to be taken into account when the relevance of specific prognostic factors is discussed.

In an initiative to develop a uniform approach to risk classification in childhood ALL, the National Cancer Institute (NCI) sponsored a concerted publication of major U.S. study groups that described the so-called NCI criteria. In this 1996 publication, the major risk groups in B-precursor (B-pc) ALL (standard and high), excluding infants, were defined by age at diagnosis and initial leukocyte count (WBC) based on criteria developed during an international workshop in Rome in 1985 *(38,39)*. Table 1 shows the proportions of standard- and high-risk patients with childhood B-pc ALL defined by NCI criteria from four consecutive ALL-BFM trials (81–90).

Continuing research on the clinical, biologic, immunologic, and genetic aspects of ALL has identified numerous features with prognostic potential, several of which have been extensively evaluated in large patient populations, although not uniformly in all subgroups. These prognostic factors include characteristics such as sex, race, CNS status at diagnosis, and

Table 2
Prognostic Factors Recommended to be Assessed in Patients with Childhood ALL

Factor	*Favorable*	*Unfavorable*	*References*
Age at diagnosis (yr)	≥1 and <10 yr	<1 or ≥10 yr	*6,15,38,39*
Sex	Female	Male	*15,16,40*
WBC count at diagnosis ($\times 10^9$/L)	<50	≥50	*6,15,38,39*
Immunophenotype	Common ALL	Pro B-ALL, T-ALL	*15, 41–43*
CNS disease[a]	No (CNS1)	Yes (CNS3)	*15, 44*
Genetic features[b]	DNA index[c] >1.16, *TEL/AML1* positivity, hyperploidy	DNA index ≤ 1.16, hypoploidy, t(9;22) or *BCR/ABL* positivity, t(4;11) or *MLL/AF4* positivity	*15, 45–54*
Early response to treatment (peripheral blood)	<1 × 10^9/L blood blasts after 7 d of induction with daily prednisone and a single intrathecal dose of methotrexate on treatment d 1	≥1 × 10^9/L blood blasts after 7 d of induction with daily prednisone and a single intrathecal dose of methotrexate on treatment d 1	*9, 15, 30, 31*
Early response to treatment (bone marrow)	<5% leukemic blasts in the bone marrow (M1) on d 7 and d 15 of induction treatment	>25% leukemic blasts in the bone marrow (M3) on d 7 and/or d 15 of induction treatment or 5–25% leukemic blasts in the bone marrow (M2 or M3) on d 15 of induction treatment	*32, 33, 34*
Remission status after induction therapy	Remission bone marrow (M1) (BFM treatment d 33)	No response to treatment exemplified through ≥5% blasts in the bone marrow (M2 or M3) after induction therapy	*15, 55*

Abbreviations: BFM, Berlin–Frankfurt–Munster (group); CNS, central nervous system; WBC, white blood cells.
[a]For patients with CNS2 status see the Risk-Adapted Therapy Stratification section
[b]Assessed by flow cytometry, cytogenetic techniques, or molecular genetic techniques.
[c]Defined as the ratio of DNA content in leukemic G0/G1 cells to that of normal diploid lymphocytes.

particular immunologic (e.g., T-cell immunophenotype) or genetic features (e.g., structural and numerical chromosomal aberrations) of the leukemic clone *(1–3)*. Table 2 shows a minimum list of prognostic variables that, are currently recommended to be assessed in childhood ALL. Because treatment strategies differ among study groups, it is difficult to make uniformly applicable statements about these factors. Therefore, most large study groups now assess the most promising of these features on a regular basis in prospective trials in order to demonstrate their prognostic strength in their corresponding patient populations. This approach allows valid comparisons of different treatments for specific patient subgroups defined by the features in question and therefore may help to define which treatment components are beneficial for which subgroup. Clearly, the success of these cooperative ventures strongly depends on the quality of communication among the large study groups and the ability to arrive at a consensus to generate the tools needed to develop a more detailed uniform approach for risk assessment in childhood ALL.

3.1. The Value of Early Response to Treatment for Risk Assessment

Several study groups have evaluated a variety of early response estimates as prognostic factors for treatment allocation in childhood ALL *(30–37)*. The BFM study group started as early as 1983 to assess the value of the so-called prednisone response *(30)*. (See Textbox 1 for a definition of this response as well as other measures of early response to treatment.) Since 1986, the BFM group has used the prednisone response for patient stratification *(9)*. Within the long-term experience of the BFM study group, including 3735 childhood ALL patients from 1983 to 1995, the in vivo response to prednisone has consistently been one of the strongest prognostic factors for the prediction of treatment outcome *(56)*. The prognostic significance of inadequate reduction of leukemic blasts in peripheral blood was confirmed in the St. Jude Total Therapy Study XI, in which the early response to multiagent remission induction therapy was evaluated retrospectively *(36)*. In that study the adverse prognostic impact of an increased cell mass was also demonstrated, but age <1 yr or >10 years was the only adverse factor identified for B-lineage patients. Children's Cancer Group (CCG) investigators have also utilized early response (as measured in the BM on d 7 and d 14 of induction) to identify patients at higher risk for failure *(34)*. In the UKALL-X, after stratification for age, gender, and WBC, the most significant prognostic factor was also early response, as measured in the BM on d 14 *(5)*. The specificity of response evaluation might vary with the composition of the induction regimen and the time of response evaluation *(34,35,57)*.

Textbox 1
Measures of early response to treatment in childhood ALL

Prednisone response (BFM study group)

In current BFM trials for ALL, therapy for all patients starts with a 7-d monotherapy with prednisone and one intrathecal dose of methotrexate on d 1. The first day of treatment is the day of the first administration of prednisone. The dosage of prednisone is increased steadily to 60 mg/m^2 daily according to leukemic cell mass and renal and metabolic parameters in order to circumvent complications of acute cell lysis. The number of leukemic blasts in the blood on d 8 is calculated from the absolute leukocyte count and the percentage of blasts in peripheral blood smears as determined by central review in the study center. The presence of ≥1000/μL blasts in the blood on d 8 is defined as *prednisone poor response*; a count of leukemic cells in blood of <1000/μL is required for the diagnosis of *prednisone good response*.

Bone marrow day 7 and day 14
(as evaluated by Children's Cancer Group, for example)

On treatment d 7 and treatment d 14 of remission induction therapy, bone marrow aspirates are obtained from the patient. The early response to therapy in the bone marrow is rate M1, M2, or M3. M1 represents a bone marrow aspirate displaying <5% residual leukemic blasts and signs of recovering hematopoesis. M2 refers to a bone marrow aspirate with the presence of leukemic blasts in the range of 5–25%, whereas and M3 rating describes all bone marrow aspirates in which the percentage of leukemic blasts exceeds 25%. Extremely hypocellular marrow aspirates are generally regarded as <5% residual blasts (M1). At both time points, an M1 rating confers a good prognosis, whereas M2 and M3 ratings are associated with a poorer prognosis. The group of patients with M2 or M3 marrows on d 7 can be further separated into patients with an intermediate or poor prognosis by using the d 14 marrow score. Those with an M2 or M3 marrow on d 14 are the subset of patients with a poor prognosis.

3.2. The BFM Experience with the Prednisone Response

In contrast to other measures of early response to treatment (Textbox 1), the morphologic evaluation of peripheral blood smears in a central setting yields highly reproducible results, whereas BM morphology is far more susceptible to bias introduced through, for example, technical variability related to the marrow aspiration procedure. Nevertheless, there are also limitations with regard to the prednisone response. One such limitation addresses the patient population with an initial leukemic blast count of <1000/μL (approx 15% of the patient population). Even though these patients, arguably, cannot be evaluated accurately for the kinetics of their leukemic cell reduction, their assignment to the group of prednisone good-responders (defined by blast counts < 1000/μL on treatment d 8) does not result in a difference in treatment results compared with "true" prednisone good-responders. In contrast, patients with very high initial blast counts and impressive leukemic cell mass reduction under prednisone (to blast counts ≥ 1000/μL on treatment d 8) may be subject to overtreatment.

Despite these valid arguments related to the definition of prednisone response, to date we have not identified a better prognostic variable applicable to the majority of patients for separating individuals with a good prognosis from those with a poor prognosis. As an example, Fig. 1A shows our most recent results with the prednisone response as a clinical tool for risk assessment and therapy stratification in a large subgroup of evaluable patients with B-precursor ALL, from the ALL-BFM 90 trial *(15)*. Whereas the 1694 prednisone good-responders had a 6-yr event-free survival rate of 81%, the 99 prednisone poor-responders only reached an event-free survival of 33% during the same period. For comparative purposes, Fig. 1B shows the subset of patients from the same patient population that had data on morphologic BM evaluation on treatment d 15 available at the reference laboratory. In addition to the advantages mentioned above of the prednisone response compared with BM analysis for the evaluation of early response to treatment, it is apparent that prednisone response is a more specific predictor of treatment outcome in ALL-BFM studies than is BM evaluation at d 15.

3.3. Prognostic Relevance of Risk Factors

With regard to the prognostic relevance of markers used for risk assessment in childhood ALL, Fig. 2 shows data from the ALL-BFM study group using a variety of variables separated by good or poor prognostic impact on treatment outcome. In a hypothetical scenario of a patient population experiencing a 20% therapy failure rate, for example, a poor prognostic marker would ideally be applicable to all patients, identifying those who will fail conventional treatment as opposed to those who can expect cure. Thus, it should test positive in 20% of the population. A comparable scenario could be hypothesized for a marker conferring a good prognosis. Although such markers with 100% sensitivity and specificity do not exist in real life, Fig. 2 exemplifies that in ALL-BFM patients, the prednisone poor response identifies 10% of the patient population, accounting for almost 30% of all events.

This excellent model of sensitivity and specificity is exceeded only by a molecular method of monitoring response to treatment, namely, by detection of minimal residual disease with, for example, a leukemic clone-specific immunoglobulin or T-cell receptor rearrangement *(29,58–65)*. Certain genetic markers such as chromosomal translocations are not displayed in Fig. 2. However, all available information is necessary to postulate the perfect risk-adapted treatment. Table 3 gives an example of such a strategy by providing information on subgroups identified by initial patient and genetic characteristics in association with prednisone response. Clearly, even within genetically or immunophenotypically defined subgroups, for example, differences in treatment responsiveness may be found (Table 3). An understanding of this heterogeneity will require a more thorough analysis of host factors.

Still, a large number of relapses appear to be unpredictable with currently available clinical, genetic, or immunologic

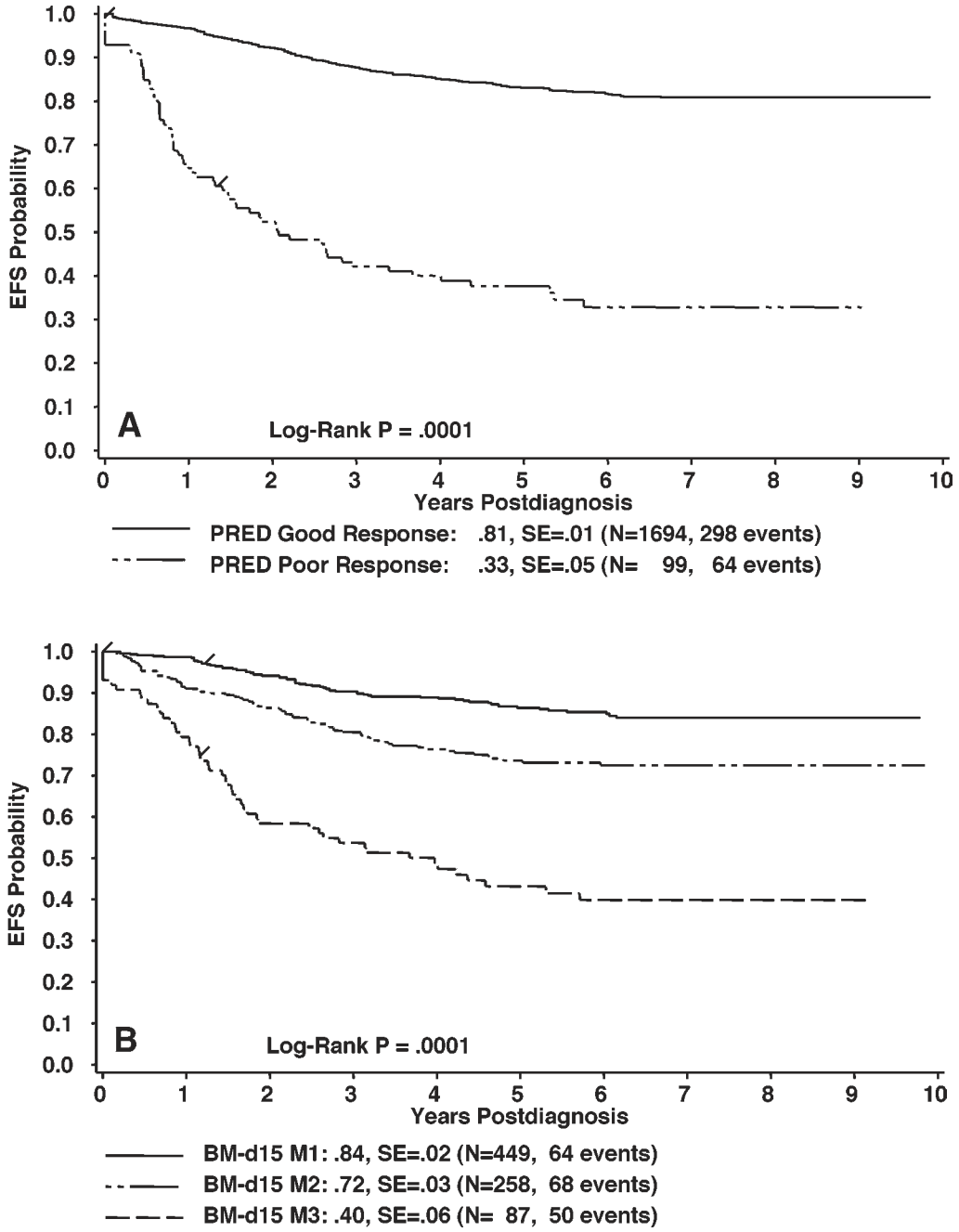

Fig. 1. Probability of event-free survival (EFS) in B-precursor cell ALL, according to response to treatment. **(A)** Response to a 7-d prednisone prephase (and one intrathecal methotrexate dose on d 1) as defined by < 1000 leukemic blasts (PRED good response) or ≥ 1000 blasts on d 8 of treatment (PRED poor response). **(B)** Response to 14 d of BFM induction (including prednisone prephase) as defined by bone marrow (BM) analysis on d 15 (centralized): M1, <5% blasts; M2, 5–25% blasts; M3: ≥25% blasts.

markers. For this large patient subset, the careful monitoring of microscopically, immunologically, or molecular genetically detectable in vivo treatment response might provide the means to target more intensive therapy to the patient at true risk of relapse *(29,33,58–65)*.

3.4. Evaluation of Treatment Response by Measurement of Minimal Residual Disease

As discussed above, conventional methods of risk classification in childhood ALL do not appear to be sufficient for identifying the patient at true risk for relapse. Even though the poor early response is highly predictive of treatment failure, most recurrences are still observed in the large group of patients with an adequate early response to treatment *(9,15)*. Therefore, based on the first results with regard to early response to treatment generated by the ALL-BFM study group and the findings from molecular detection of minimal residual leukemic disease, a prospective minimal residual disease (MRD) study was initiated by the International BFM study group in 1991 *(61)*. In that study, patients from Austria, Germany, Italy, and the Netherlands were enrolled. Treatment was

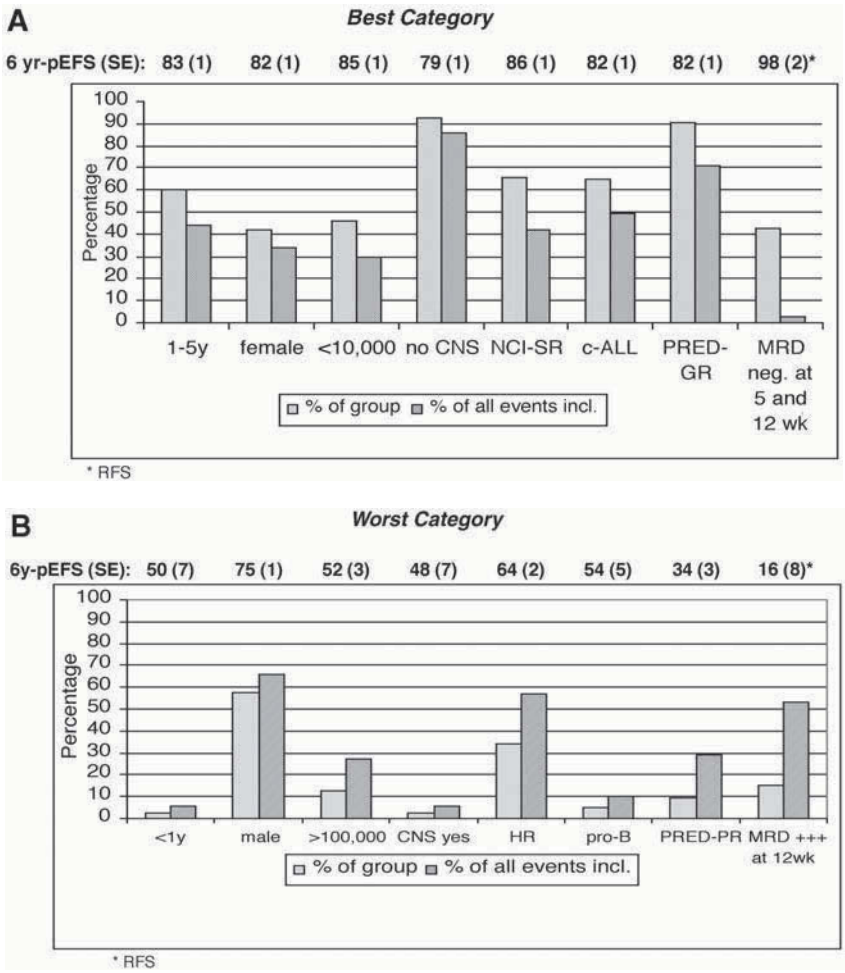

Fig. 2. The prognostic relevance of important presenting features and response in childhood ALL. **(A)** Best risk category: Except for the early negativity of minimal residual disease (MRD) the specific prognostic relevance of these variables is low. **(B)** Worst risk category: The highest proportion of recurrences is found among patients with a prednisone poor response (PRED-PR) and high MRD positivity at 12 weeks (= MRD +++ at 12 wks). NCI-SR, standard risk by NCI criteria; PRED-GR, prednisone good response; MRD, minimal residual disease; EFS, event-free survival; HR, high-risk by NCI criteria; PRED-PR, prednisone poor response; EFS, event-free survival; CNS, central nervous system.

Table 3
Outcome by Prednisone Good Response and Prednisone Poor Response in Specific Patient Subsets

	Prednisone good response		*Prednisone poor response*		
Subset	*No. of patients*	*% EFS ± SE*	*No. of patients*	*% EFS ± SE*	*Reference*
NCI risk group[a]					
Standard	1324	87± 1	48	45 ± 7	*15*
High	564	73 ± 2	143	31 ± 4	
Immunophenotype					
Pro-B ALL	80	68 ± 6	19	0	*15*
Common ALL	1274	84 ± 1	67	46 ± 6	
Pre-B ALL	338	79 ± 2	13	31 ± 13	
T-ALL	180	78 ± 3	101	32 ± 5	
Genetic aberrations					
t(9;22) or *BCR/ABL* positive	37	55 ± 8	20	10 ± 7*66*	*66*
Infants					
All infants	78	53 ± 6	27	15 ± 7	*67*
Infants with 11q23 rearrangement	17	41 ± 12	11	9 ± 9	
Infants t(4;11) or *MLL/AF4* positive	9	33 ± 16	7	0 ± 0	

Abbreviations: EFS, event-free survival; NCI, National Cancer Institute.
[a]For explanation of NCI risk groups see section 3 of this chapter. Data from recent ALL-Berlin-Frankfure-Münster (BFM) trials.

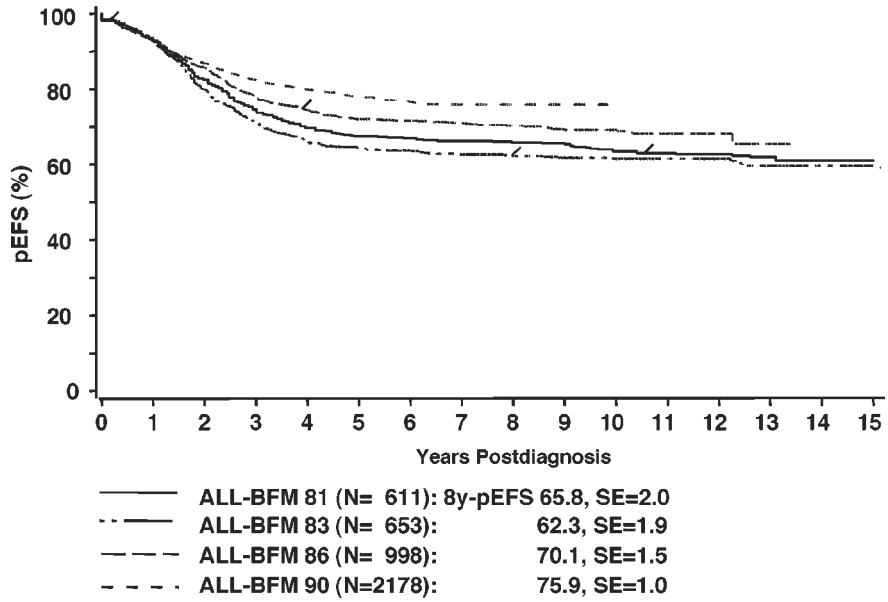

Fig. 3. Event-free survival (EFS) in four consecutive trials of the ALL-Berlin–Frankfurt–Münster (BFM) study group.

based on the strategy of the ALL-BFM 90 protocol *(15)*. The results of this multicenter trial on MRD showed that the individual response to treatment, as measured by MRD analysis using PCR-based detection of leukemic clone-specific immunoglobulin and/or T-cell receptor gene rearrangements, was by far the strongest predictor of outcome. For the first time, it was possible to identify patients with basically no risk of relapse and to define patients who had a >80% probability of relapse while on current treatment protocols. The remaining intermediate-risk patients were defined by measurable but decreasing levels of MRD and were found to have a prognosis of approx 75% relapse-free survival. Similar results on the value of MRD in childhood ALL could also be demontrated by others *(62–64)*. With regard to methodologic issues, it was shown that flow cytometric analysis of minimal residual disease by detection of specific antigen patterns of the leukemic clone yields sensitive and reliable results comparable to those of PCR-based approaches *(60,64,65)*.

3.5. How Will the Analysis of Minimal Residual Disease Contribute to Treatment Success in Childhood ALL?

The first important aim will be to confirm the prognostic value of MRD in large prospective therapy studies. It will be possible to analyze MRD with a variety of different tools and it will become clear whether these tools complement each other or have advantages and disadvantages with regard to specific clinical and methodologic, but also logistic and financial issues. Results derived from such studies will improve our understanding of the importance of disease kinetics with regard to outcome in childhood ALL. Solid new molecular definitions for remission and relapse, which may eventually replace the traditional morphology-based classifications, are obviously difficult to construct *(68)*. The more sensitive techniques will demonstrate a large variability in the kinetics of the treatment response, dependent not only on leukemic subtype but also on the number and dosage of drugs being used. More important, MRD analysis at predefined time points with standardized methodology may be used for stratification.

A large international study group formed by the Italian Association for Pediatric Hematology and Oncology (AIEOP) and the German–Austrian–Swiss ALL-BFM Study Group has decided to replace conventional risk variables completely with response evaluation (on the basis of either prednisone response and/or PCR-based MRD detection at two highly informative time points) for selecting postconsolidation treatment intensity. That trial will not only allow controlled treatment reduction in MRD low-risk patients but will also apply treatment intensifications to MRD-intermediate- and high-risk patients. Only if treatment results after such MRD-based reassignment of patients demonstrate that less intensive therapy is uniformly efficacious in low-risk patients and that more intensive or alternative therapy can reduce the number of recurrences in patients identified by MRD as being more resistant, will there be justification to introduce that technique as an essential tool for follow-up. There will certainly be a strong desire among clinicians to use this more sensitive test more frequently throughout therapy, in particular if relapse is suspected. This would produce a large number of treatment deviations. The more heterogeneous these deviations, the more difficult it will be to derive prognostic information from future trials.

4. TREATMENT

In childhood ALL overall cure rates of 65% to almost 80% have been achieved by application of intensive multiagent chemotherapeutic regimens *(1–17,56)*. Figure 3 displays updated results (event-free survival) of four trials of the ALL-BFM study group performed from 1981 to 1995. With the exception of trial ALL-BFM 83, a steady increase in prognosis was observed *(15,56)*. Modern regimens consist of at least four elements including (1) an induction phase aiming at an

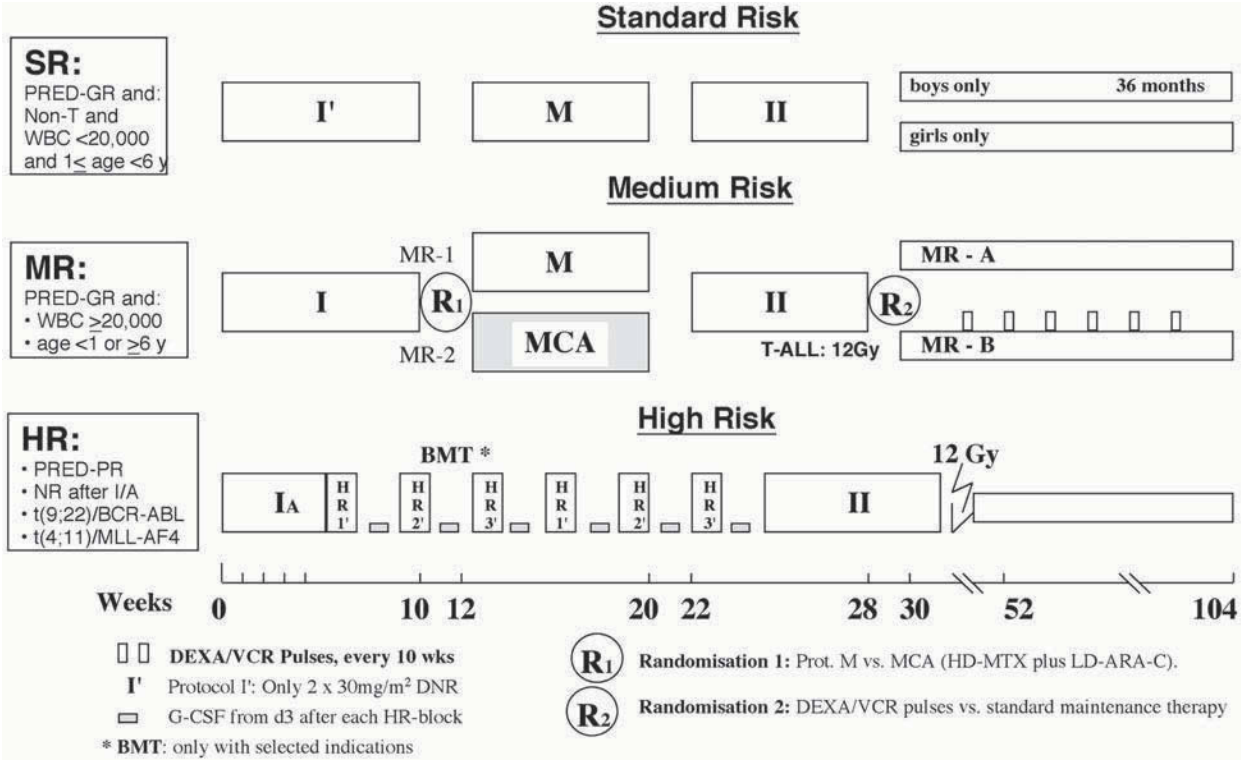

Fig. 4. Outline of treatment strategy applied in the ALL-Berlin–Frankfurt–Münster (BFM) 95 study (1995–2000), in which patients were assigned to standard-risk (SR), medium-risk (MR), and high-risk (HR) subgroups. PRED-GR, prednisone good response; PRED-PR, prednisone poor response; DEXA, dexamethasone; VCR, vincristine; DNR, daunorubicin; HD-MTX, high-dose methotrexate; LD-ARA-C, low-dose cytarabine; BMT, bone marrow transplantation.

initial remission induction within approximately 4–6 wk through the use of multiple cancer chemotherapeutic drugs; (2) a consolidation segment to eradicate residual leukemic blasts in patients who are in remission by morphologic criteria; (3) extracompartment therapy such as CNS preventive therapy, and (4) a maintenance period to stabilize remission further by suppressing reemergence of a drug-resistant clone through continuing reduction of residual leukemic cells.

In the second half of the 1970s, the ALL-BFM study group introduced an additional treatment element, a so-called reinduction or delayed reintensification phase *(16,69)*. For certain patient populations, preventive or therapeutic cranial radiotherapy may be needed as an additional treatment component to target leukemic cells in the CNS specifically. A last important introductory issue addresses the definition of what is called complete remission (CR) and relapse *(9,15)*: CR is defined as the absence of leukemic blasts in blood and CSF, <5% lymphoblasts in marrow aspiration smears, and no evidence of localized disease. Relapse is defined as recurrence of lymphoblasts or localized leukemic infiltrates at any site.

4.1. Risk-Adapted Therapy Stratification

The intensive and potentially life-threatening multimodal treatment regimens used in ALL are usually tailored to a patient's individual risk profile, as described in the risk assessment section of this chapter. The various risk assessment procedures applied by different study groups mainly translate into therapy stratification into one of three risk groups (standard/low, intermediate, high) *(1–3,6,9,15)*. As an example of a risk-adapted modern clinical protocol, Fig. 4 shows an outline of the treatment strategy applied in the ALL-BFM 95 study (1995–2000), in which patients were assigned to standard-risk (SR), medium-risk (MR), and high-risk (HR) subgroups. The main criteria for stratification were the early response to treatment (prednisone response), initial WBC count, and age at diagnosis. Additional criteria included the presence of T-cell immunophenotype, a *BCR/ABL* rearrangement or t(9;22) translocation, and an *MLL/AF4* rearrangement or t(4;11). Textbox 2 shows the exact stratification criteria by risk group. As can be seen in Fig. 4, all patients who did not qualify for the high-risk therapy received induction protocol I (with a reduced anthracycline dose in SR patients, protocol I'), consolidation/extracompartment protocol M, reinduction (delayed intensification) protocol II, and maintenance therapy. High-risk patients were treated with a shorter induction and continued on a more intensive rotational consolidation schedule consisting of three different 6-d-long pulses of high-dose chemotherapy (HR–1, –2, and –3), which were repeated twice and followed by reinduction (delayed intensification) protocol II. Maintenance therapy was initiated 2 wk after the end of reinduction (protocol II). Drugs in maintenance therapy were orally administered daily 6-mercaptopurine and methotrexate once per week. Total therapy duration was 24 mo for all patients except for boys in the SR subgroup, who received 36 mo of maintenance therapy (Fig. 4).

4.2. Remission Induction

Contemporary treatment approaches for childhood ALL aim at an initial remission induction within approx 4–6 wk through the use of multiple cancer chemotherapeutic drugs *(1–17)*. In most of the study groups, this is generally achieved through the systemic application of three drugs (glucocorticoid, vincristine, and L-asparaginase) to which an anthracycline may be added as a fourth drug. Applying such a treatment strategy, >95% of the childhood ALL patients usually achieve remission (in the ALL-BFM 90 study, 98.3% CR rate); the remaining 5% of patients will either have died of treatment- or disease-related complications or display nonresponsive disease *(9,15,17,55)*. The latter group includes patients that will achieve only delayed remission or show resistant disease. Because of the poor prognosis of this minor nonresponsive patient population, alternative therapeutic approaches should be considered early during the disease process.

Within the ALL-BFM strategy, remission induction is initiated with a 7-d monotherapy with orally administered prednisone (and one dose of intrathecal methotrexate on d 1), which is complemented by intravenous application of three additional drugs (vincristine, daunorubicin, and l-asparaginase) starting on treatment d 8. The prolonged initiation of induction therapy through the 7-d prednisone prephase is particularly useful in avoiding complications related to extensive tumor cell lysis. In the BFM group, this first phase of induction treatment is subsequently followed by an early intensification phase including intravenous cyclophosphamide and cytarabine, intrathecal methotrexate, and oral 6-mercaptopurine. The outlined strategy of prolonged induction/early intensification proved to be successful in several BFM trials as well as trials performed by other study groups *(4,6,9,15–17,56,69–71)*.

4.3. What Is the Optimal Number of Drugs Used and What Is the Best Choice of a Glucocorticoid During Induction Treatment?

In contrast to adult ALL, for which a four-drug remission induction therapy including an anthracycline almost seems mandatory, the necessity of such a four-drug induction regimen in specific subgroups of pediatric ALL is subject to debate. It is unclear whether addition of an anthracycline to a three-drug induction regimen is of benefit to certain low- or intermediate-risk groups. In a study of the Children's Cancer Group for intermediate-risk patients, it appeared that patients 10 years of age or older fared best if they received the full four-drug BFM induction/consolidation and reintensification (along with cranial radiotherapy), whereas children younger than 10 years of age fared equally well if induction contained only prednisone, vincristine, and asparaginase *(70)*. The dose intensity of the induction phase can also have a major impact on the overall results, as was demonstrated by the result of the ALL-BFM 83 study, which was significantly inferior compared with the ALL-BFM 86 and 90 studies *(9,15,30)*. In the ALL-BFM 83 study, the induction phase was 14 d longer than in the ALL-BFM 90 study with the same cumulative dose of the four drugs used (prednisone, vincristine, daunorubicin, and L-asparaginase).

In some regimens, only two or three drugs are used in the first 4 wk of treatment: in the Dutch study VI for non-high-risk patients, treatment is initiated with vincristine and dexamethasone only, before L-asparaginase is added *(71)*. In the COALL regimen, the first 4 wk of treatment also omit L-asparaginase. Preliminary data from that group indicate that the number of drugs as much as the time used for induction therapy has an impact on the response (as detected by analysis of MRD) but not necessarily on the final outcome *(72)*. Addition of high-dose methotrexate early in a three-drug induction regimen, as shown in a limited number of patients by the DFCI Consortium, can improve disease control but also strongly enhances toxicity *(73)*. The choice of the corticosteroid for optimal induction therapy is still being debated *(74)*. Dexamethasone appears to have a stronger antileukemic effect, which can be demonstrated in vitro *(75)*. However, both in vivo and in vitro resistance of ALL cells to prednisolone is also associated with increased resistance to dexamethasone *(37)*. One study in the Netherlands (Dutch Study VI) for non-high-risk patients demonstrated the feasibility of 4 wk of dexamethasone (at 6 mg/m^2/d) when combined only with vincristine, whereas a pilot study performed by the DFCI group indicated severe complications, including toxic deaths, when dexamethasone was combined with doxorubicin, vincristine, and L-asparaginase *(71,76)*.

Textbox 2
Stratification criteria in trial ALL-BFM 95 by risk group: standard, medium, and high

SR: prednisone good response, initial WBC < 20×10^9/L, age at diagnosis ≥1 to <6 yr, no HR cytogenetics, and no T-ALL (all criteria to be fulfilled)

MR: prednisone good response, no HR cytogenetics, and one of the following: initial WBC ≥ 20×10^9/L, or age at diagnosis <1 or ≥6 yr

HR: prednisone poor response, prednisone good response but ≥5% marrow blasts on treatment d 33 (M2 or M3 marrow), t(9;22) or *BCR/ABL* positivity, t(4;11) or *MLL/AF4* positivity

4.4. Consolidation/Extracompartment Therapy

Eradication of residual leukemic blasts in patients who are in remission by morphologic criteria is the primary aim of consolidation treatment. Consolidation treatment is necessary, as patients successfully induced into remission but not given additional treatment usually relapse within months *(1)*. Today, most study groups use 6-mercaptopurine and methotrexate to maintain remissions. Only a few groups use continous infusion cycles of high-dose methotrexate (combined with leucovorin rescue) for consolidation treatment. The contribution of high-dose methotrexate in consolidation/extracompartment therapy is probably largely owing to its CNS protective effect, as cytotoxic methotrexate levels are also achieved in the CSF during high-dose methotrexate application *(77,78)*. Another systemically administered drug that is discussed in the context of CNS disease prevention is dexamethasone, which was shown to be superior to prednisolone *(71,79)*. The importance of this finding is currently being evaluated by several study groups. For

further extracompartmental disease prevention, repeated intrathecal injections of methotrexate throughout the treatment period and cranial radiotherapy in defined subsets of patients are used *(6,8,9,15,80)*. Instead of the sole application of intrathecal methotrexate, some study groups add additional drugs to this treatment (a glucocorticoid, either hydrocortisone or prednisolone, and cytarabine, mostly restricted to high-risk patients) *(15,81)*. It remains unclear whether triple-drug intrathecal injections are of any benefit to any patient subset. Treatment of CNS disease is covered extensively in other chapters of this book.

In trial ALL-BFM 81 *(82)*, low- and intermediate-risk patients were randomized to compare the efficiency of 18-Gy cranial radiotherapy vs intermediate-dose methotrexate (0.5 g/m^2) given as four 24-h infusions, together with intrathecal methotrexate every 2 wk during consolidation. The higher incidence of relapses found in those patients who did not receive cranial radiotherapy was owing to the higher number of relapses with CNS involvement: 19 relapses in one randomization arm vs three relapses in the other. Thus, this first attempt of the BFM study group to replace cranial radiotherapy by systemic methotrexate was not successful *(83,84,56)*. However, subdividing the group of "standard risk" ALL patients according to the initial cell mass revealed that low-risk patients were in fact quite well protected from CNS-related relapses with intermediate-dose methotrexate without cranial radiotherapy (1.6% isolated and 3.2% combined CNS relapses). Nevertheless, the rate of relapses after long-term observation of low-risk patients from the ALL-BFM 81 trial demonstrated an advantage for the irradiated subset of patients (all relapses 12.9% vs. 22.2%).

In the subsequent ALL-BFM 83 trial, a strong impact of intensive reintensification on the rate of systemic and extramedullary relapses was found in a randomized study for low-risk patients *(56,83)*. All patients received intermediate-dose methotrexate during consolidation, but low-risk patients were then randomized to receive or not receive postconsolidation reinduction therapy. This was an attempt to decrease the overall toxicity of ALL treatment and to reduce the anthracycline dose by 30% in the group of patients with the lowest risk for relapse. It was also decided that patients in both treatment arms would not be irradiated under the protection of intermediate-dose methotrexate. The number of isolated CNS relapses was low in both groups, but the rate of combined CNS and BM relapses, and especially of isolated systemic relapses, was significantly higher in patients who had not been exposed to reinduction therapy. When the results were compared with the corresponding subset of patients from the ALL-BFM 81 trial, the rate of combined CNS/BM relapses was also three times higher in patients not receiving reinduction therapy. This provided evidence that reinduction therapy is important not only for systemic but also for extramedullary disease control *(56,78)*.

The first reduction of preventive cranial radiotherapy from 18 to 12 Gy in patients other than those with low-risk ALL was performed on a randomized basis in the ALL-BFM 83 trial *(30,56,78)*. Intermediate-risk patients were treated with either 12 or 18 Gy cranial radiotherapy, with patients receiving intermediate-dose methotrexate during consolidation and a total of eight intrathecal methotrexate injections throughout treatment. With regard to CNS protection, the two cranial radiotherapy regimens were equally effective. There was a slightly higher rate of systemic relapses in patients treated with 12 Gy, but the difference was not statistically significant. Comparison of these intermediate-risk patients with the corresponding subset of patients from the ALL-BFM 81 trial, who had received systemic methotrexate but not presymptomatic radiotherapy, confirmed the large difference in the number of CNS-related relapses found in the randomized comparison in the earlier trials, indicating that intermediate-dose methotrexate without cranial radiotherapy does not provide adequate CNS protection *(56,78,84)*.

In the subsequent ALL-BFM 86 and ALL-BFM 90 trials *(9,15)*, additional reduction of cranial radiotherapy was performed under the protection of intensified intrathecal methotrexate therapy as well as systemic application of high-dose methotrexate (5 g/m^2 × 4 in consolidation). In the ALL-BFM 86 trial, high-risk patients were treated with 18 instead of 24 Gy. In the ALL-BFM 90 trial, preventive cranial radiotherapy was only 12 Gy for intermediate- and high-risk patients. The results of these two trials showed that identical subsets of intermediate-risk patients defined by prednisone good response and B-precursor ALL had CNS-related relapse frequencies of <3%. In the ALL-BFM 90 trial, even in cases of B-precursor ALL with an increased initial cell mass but adequate early response to prednisone, no increase in CNS-related relapses could be demonstrated after the dose of preventive cranial radiotherapy had been decreased from 18 to 12 Gy *(15)*. Also, in T-cell ALL patients in both the ALL-BFM 86 and 90 trials, the relapse incidence could be reduced, especially among prednisone good responders, by the introduction of high-dose methotrexate in consolidation therapy *(9,15)*. In the ALL-BFM 86 trial, only 1.4% of isolated and no combined CNS/bone marrow relapses were observed among 81 CNS leukemia-negative patients with T-cell ALL; the overall relapse incidence was 14.8% for this subset. Intermediate-risk patients with prednisone good response received 12 or 18 Gy for CNS protection, depending on the leukemic cell mass estimate (so-called BFM risk factor). The 6-year event-free survival (EFS) rate of 73 ± 4% for T-cell ALL patients in the ALL-BFM 86 trial is one of the best results ever reported for this disease subtype. A similar strategy applied to T-cell lymphoblastic non-Hodgkin's lymphoma yielded an EFS rate of >90% in the NHL-BFM 90 trial *(85)*. In the ALL-BFM 90 trial, when only 12 Gy were used, 161 T-cell ALL patients with a prednisone good response had an overall incidence of relapses of 11.8%; relapses with CNS involvement have been diagnosed in 3.2% of patients. Thus, 12 Gy of preventive cranial radiotherapy for T-cell ALL patients with a prednisone good response provided effective control of systemic and CNS recurrence *(15)*.

Detection of the prognostic significance of the in vivo response to prednisone in the ALL-BFM 83 trial *(30)* provided a new tool for identifying very early and easily patients who are at significantly higher risk for systemic and CNS relapse. In the ALL-BFM 86 trial, this finding was utilized for the first time for stratification *(9)*. Only the small group of high-risk patients mainly qualified by their inadequate corticosteroid response

(10% of the total study population) had an increased incidence (11.6%) of relapses with CNS involvement. The overall outcome and also the cumulative incidence of CNS relapses (CI CNS) were not significantly improved in patients with a prednisone poor response in the ALL-BFM 86 trial vs those of trial 83: 8-yr EFS, 37.6 ± 7.0% in trial 83 (CI CNS, 18.2 ± 6.9%) vs 46.2 ± 5.2% (CI CNS, 13.7 ± 4.2%) in trial 86 *(56)*. The introduction of a modified approach to the high-risk group in the ALL-BFM 90 trial, utilizing a postinduction series of intensified consolidation elements (containing high-dose methotrexate, high-dose-cytarabine, and nine intrathecal doses of intrathecal triple therapy), decreased the number of relapses with CNS involvement significantly, even though the dose of cranial radiotherapy was decreased to 12 Gy in patients without CNS involvement. On the other hand, this approach was not successful in reducing the rather high rate of systemic relapses: 8-yr EFS for patients with a prednisone poor response, 31.8 ± 3.4%; (CI CNS, 5.3 ± 2.4%) *(15,56)*.

With regard to the influence of methotrexate dose during consolidation/extracompartment therapy on the incidence of testicular relapse in boys with ALL, 1144 boys with newly diagnosed ALL, enrolled in ALL-BFM 81, 83, or 86, were retrospectively evaluated for the influence of methotrexate on the testicular relapse-free interval *(86)*. As explained earlier, the basic treatment design was similar in all three trials. Intravenous methotrexate (0.5 g/m^2/24 h × 4) was used in the ALL-BFM 81 trial only in the group of standard-risk patients who received no cranial radiotherapy for CNS prophylaxis. Four courses of intermediate-dose methotrexate were introduced for all patients in the ALL-BFM 83 trial, and were replaced by high-dose methotrexate (5.0 g/m^2/24 h × 4) in the ALL-BFM 86 trial. The median observation time at the time of analysis was >9 yr. We observed that the cumulative incidence of isolated testicular relapses was significantly higher in the group receiving cranial radiotherapy compared with the intermediate-dose and high-dose methotrexate groups (6.7 vs 2.5% and 2.3%, $p = 0.02$ and 0.01, respectively). High-dose methotrexate did not lower the rate of isolated testicular relapses any further.

4.5. Who Needs Preventive Cranial Radiotherapy?

Most CNS relapses of ALL are observed within 24 mo of the initiation of treatment, indicating the importance of effective and early CNS prevention. Because cranial radiotherapy does cause more acute clinical side effects and might cause secondary malignant glioma, it was hoped that this treatment could be eliminated from ALL therapy, at least for low- and intermediate-risk patients. This possibility was tested by randomized evaluation of patients in the ALL-BFM 81 trial but failed as described above *(82,83,56)*. Adjustments of CNS-directed chemotherapy in several consecutive clinical trials performed thereafter have led to a successful strategy in which preventive radiotherapy is restricted to a well-defined patient group with an increased risk for CNS or systemic recurrence. In our opinion, this well-defined patient subset in the BFM study group is represented by all patients 1 yr of age or older with T-cell ALL (intermediate-risk and high-risk) and all high-risk ALL cases. (*See* Textbox 2 for stratification criteria in ALL-BFM 95.) With regard to intermediate-risk T-ALL, an intergroup analysis performed by the Italian ALL study group AIEOP and the BFM study group revealed the importance of cranial radiotherapy in cases of prednisone good-responding T-cell ALL for the prevention of CNS as well as systemic relapses *(87)*.

The main difference between these regimens was the lack of cranial radiotherapy in the Italian study, which was replaced by nine doses of intrathecal methotrexate/cytarabine/prednisolone (intrathecal triple therapy) during maintenance. T-cell ALL patients treated on AIEOP 91 had two times more systemic and five times more CNS relapses than patients treated on BFM 90. When the EFS probability in T-cell ALL patients with a high WBC count (>100 × 10^9/L) was compared, the largest difference resided in the probability of EFS at 3 yr for the patients in AIEOP 91: 17.9% compared with 81.9% in BFM 90 *(87)*. Another study for intermediate-risk patients of the CCG demonstrated that extended intrathecal methotrexate therapy is as effective as cranial radiotherapy if systemic therapy comprises a more intensive regimen with delayed intensification *(80)*. This study group also demonstrated that in high-risk patients no significant difference in outcome can be found between cranial radiotherapy and intrathecal methotrexate for CNS prevention. The latter treatment provided less effective CNS control but better protection from marrow relapse *(88)*. A Dutch study using the BFM regimen from the ALL-BFM 86 trial was successful in preventing CNS recurrences without the use of cranial radiotherapy *(9,17)*.

Whether long-term toxicity will also be diminished by this new regimen, in particular with respect to the development of secondary malignancies, remains to be determined. Within the BFM experience, ALL-BFM 90 was the first large trial for childhood ALL in which no patient subset received >12 Gy of preventive cranial radiotherapy and in which the cumulative incidence of CNS-related recurrences at 6 yr was only 4% *(15,56)*. With regard to the late effects of high-dose methotrexate, a critical comparison between patients treated with chemoprophylaxis based on systemic high-dose methotrexate and intrathecal methotrexate (or intrathecal triple therapy) and those treated with preventive radiotherapy, or combinations of both, is needed to settle the issue of which regimen has less long-term toxicity *(89,90)*. The strategy chosen in ALL-BFM 90 (12 Gy of preventive cranial radiotherapy and limited intrathecal methotrexate chemotherapy) might offer a reasonable compromise. However, because of the oncogenic potential of radiation, the ALL-BFM study group has decided to evaluate further the elimination of preventive cranial radiotherapy for patients at low risk for CNS relapse.

4.6. Reinduction

Reinduction or delayed intensification of childhood ALL treatment was introduced by the BFM group in ALL-BFM 76 *(69)*. In the second half of the 1970s, this new therapeutic approach was limited to high-risk patients who were characterized by their large leukemic cell mass (mainly based on an initial WBC count of >25 × 10^9/L). The timing of reinduction was either directly after induction or at 5 mo after diagnosis. From these studies it was learned that patients receiving this type of treatment (protocol II) fared significantly better with regard to outcome then did patients from ALL-BFM 70, in particular if reinduction was delayed: the 10-yr EFS rate for these patients was 70 ± 5% (delayed) vs 60 ± 5% (given shortly

after induction) vs 38 ± 4% in ALL-BFM 70 (no reinduction). Therefore, overall outcome was significantly better in BFM 76/79 ($n = 321$) than in the first BFM study: 10-yr EFS of 67 ± 4% vs 54 ± 5% in BFM 70 ($n = 119$) *(83)*. Figure 4 illustrates how reinduction treatment (protocol II) is incorporated into a modern clinical treatment protocol, the treatment strategy of ALL-BFM 95, in which reinduction or delayed intensification treatment is given 2–3 wk after completion of consolidation/extracompartment therapy protocol M/MCA. Similar to induction protocol I, two treatment phases (induction and intensification) can be distinguished in protocol II: phase 1 includes systemic applications of dexamethasone (10 mg/m^2/d × 21) as a glucocorticoid, vincristine, L-asparaginase, and doxorubicin. Vincristine and doxorubicin were given in study 76 only three times, compared with four times in study 79. Phase 2 of reinduction comprises cyclophosphamide, 6-thioguanine, and cytarabine.

The first randomized clinical trials that proved the value of reinduction for the successful therapy of non-high-risk childhood ALL were conducted in parallel by the BFM group and the Children's Cancer Group in the first half of the 1980s *(30,69,83,70)*. In ALL-BFM 83, 126 patients in the low-standard-risk group (low leukemic cell mass and absence of mediastinal mass or CNS disease) were randomized to receive or not receive reintensification with so-called protocol III after interim maintenance with intermediate-dose methotrexate. Protocol III is similar to protocol II but comprises only two doses of vincristine and doxorubicin each (instead of four), only 2 wk of dexamethasone, and no cyclophosphamide. Patients did not receive cranial radiotherapy. Patients treated on protocol III showed a significantly better EFS, mainly owing to the lower incidence of systemic recurrences: 8-yr EFS, 81.8 ± 5.2% vs 58.3 ± 6.1% ($p = 0.0016$). In addition, patients receiving reintensification had a cumulative incidence of CNS recurrences of 3.8 ± 3.0% compared with 12.3 ± 4.4% in patients treated without reinduction ($p = 0.07$ by Gray's test) *(56)*.

In ALL-BFM 86, a very similar observation was made in a nonrandomized comparison between standard-risk patients, defined by a low leukemic cell mass (BFM risk factor < 0.8) and the absence of mediastinal or CNS involvement, as in ALL-BFM 83, but also by a prednisone good response, who were treated in the first part of ALL-BFM 86 without reinduction, and patients treated with the so-called reinduction protocol II after amendment of the protocol *(9,56)*. Despite the use of high-dose methotrexate during consolidation in that study, reinduction with protocol II had a major impact on the number of relapses. This was also found in the Dutch trial Study 7 *(18)*. The difference in relapse incidence between these patient groups was caused by systemic relapses and not to the number of CNS relapses. Interestingly, a recent randomized study by the CCG, applying an augmented BFM protocol in high-risk patients with a slow initial response, showed that intensified consolidation and further reinduction or double-delayed reintensification can further improve outcome for this patient subset, even though the effect appeared to be limited to patients younger than 10 years of age *(91)*. Unfortunately, this approach was associated with a high incidence of avascular bone necrosis.

4.7. Maintenance

Maintenance treatment aims at a further stabilization of remission by suppressing the reemergence of a drug-resistant clone through consistently reducing the pool of residual leukemic cells. The current BFM gold standard of maintenance therapy consists of 2 or 3 yr (only low-risk boys) of treatment with daily oral mercaptopurine and weekly oral methotrexate. On BFM protocols, dose adjustments of 6-mercaptopurine and methotrexate are made according to WBC count (target range 2–3 × 10^9/L). On protocols of other study groups, additional pulsed applications of vincristine and a glucocorticoid are administered *(92)*. It is important to note that reduction of maintenance to <2 years was associated with an increased frequency of leukemic relapses *(83,93)*.

4.8. What Is the Optimal Duration of Maintenance Treatment?

In BFM studies, maintenance therapy was based only on oral methotrexate (20 mg/m^2 once a week) and oral 6-mercaptopurine (50 mg/m^2/d). Intrathecal treatment was not used in maintenance therapy, and vincristine/steroid pulses were used only in study 76/79 *(83)* and in a recent international study for intermediate-risk patients. Total duration of treatment was investigated by randomization in ALL-BFM 81 and 83 *(83,56)*. A clear advantage of 24 mo of total treatment duration was evident, resulting in an extension to 24 mo of maintenance in the subsequent study, ALL-BFM 86. Similar results have been reported before by others. An updated evaluation of ALL-BFM 81 and 83 demonstrated at 8 yr an estimated disease-free survival of 77.3 ± 2.3% for patients randomized for 24 mo ($n = 375$) and of 71.2 ± 2.4% for patients randomized for 18 mo ($n = 389$). The log-rank test did not reveal a significant difference ($p = 0.11$) due to the number of late events that occurred more than 10 yr from diagnosis *(56)*. The same result was found if the outcome was analyzed for each trial separately. If the cumulative incidence of CNS-related and other events was calculated at 10 yr from randomization, no difference for CNS recurrences ($p = 0.8$) but a trend for difference for other relapses was found, favoring 24 mo ($p = 0.07$). Also, if the test for a difference in Kaplan–Meier estimates at 10 yr was applied, a significant difference favoring 24 mo was found ($p = 0.025$).

With our current knowledge, we conclude that within the BFM experience, 24 mo of maintenance therapy are warranted. Although it clearly seems disadvantageous to shorten maintenance treatment, whether or not extended maintenance of up to 3 yr would offer any beneficial effect in the context of BFM treatment strategies remains to be evaluated, particularly in patients of male gender. This question is currently under investigation. Other issues that have to be resolved in the future include differences in requirements for maintenance therapy in specific childhood ALL patient subsets (e.g., those defined immunophenotypically or genetically).

4.9. Bone Marrow Transplantation

Allogeneic BM transplantation from a matched related donor has been shown to improve the survival of children with ALL in second remission *(94,95)*. The main reasons for this improvement are most probably further intensification of treat-

ment and the graft-versus-leukemia effect. However, with decreasing transplant-related mortalities through progress in the understanding and management of graft-versus-host disease and the graft-versus-leukemia effect, as well as increasing numbers of registered donors, allogeneic BM or peripheral stem cell transplantations are more and more becoming a therapeutic option for specific high-risk patient subsets with ALL in first remission. Because the number of well-designed randomized clinical trials on the value of BM or peripheral stem cell transplantation in patients with ALL in first remission is small, it will be a major task of current and future trials to identify clearly those patient subsets that truly benefit from these therapeutic approaches compared with innovative high-dose chemotherapy regimens *(96,97)*.

4.10. Which Patients Should Receive Bone Marrow Transplantation in First Complete Remission?

Current BFM guidelines restrict matched related or unrelated donor BM or peripheral stem cell transplantation in first complete remission to specific subsets of high-risk patients. These guidelines include patients not in remission at the end of induction treatment (M2 or M3 marrow at treatment d 33), patients with the nonrandom chromosomal translocations t(9;22) or t(4;11) (or positivity for the respective fusion RNAs: *BCR/ABL* and *MLL/AF4*) as well as prednisone poor responders with a T-cell or pro-B-cell immunophenotype. However, considering the risk of transplant-related mortality as well as late treatment-related morbidity, it will be important in the future to develop strategies for the identification of "highest-risk" patients within the high-risk subset of children with ALL. One potential approach is shown in Table 3, which presents the EFS of patients with high-risk features, such as age <1 year or t(9;22) separated by initial response to treatment. A combined analysis of initial patient or genetic characteristics with early response to treatment can further distinguish patients with a very high risk of relapse. Analysis of MRD will probably further improve the development of eligibility criteria for transplantation.

4.11. Toxicity and Supportive Care

Quality of treatment will increasingly be the focus of attention and will be tested for its impact on the quality of life, as defined by short- and long-term toxicity. This issue has become more important as the best major study groups have reached comparable rates of long-term EFS. Supportive care, mainly through prevention of infectious complications such as cotrimoxazol for *Pneumocystis carinii* pneumonia prophylaxis, antimycotic treatment of mucous membranes, and prophylaxis for varicella-zoster virus infection, as well as uncompromised treatment of potential bacterial and/or fungal infections, has contributed in part to the above-described increases in event-free survival. Other important aspects of supportive care include appropriate hydration regimens and medications accompanying induction therapy and certain systemic chemotherapeutic applications (e.g., high-dose methotrexate and cyclophosphamide) to prevent complications from initial cell lysis and organ malfunction through drug toxicity. Finally, an adequate supply of blood components and maintenance of an adequate nutritional status are essential in the management of children with ALL, as is appropriate psychosocial support for the patient and the family.

Early mortality in ALL-BFM trials 83–90 ranged from 0.3 to 1.7% of patients, with the main causes for treatment-related fatalities being infections during neutropenia, occasionally combined with organ dysfunction *(15)*. With regard to post-remission toxicity, fatality rates of 1.3 and 1.6% were noted in ALL-BFM 86 and 90, respectively *(15,55)*. These fatalities were mainly owing to infectious complications but also involved bleeding and organ failure. The described mortality observed in BFM trials is comparable to mortalities observed in trials by other study groups *(5–8,14,17,56,70)*.

With overall improvements in survival, the long-term adverse effects of treatment have become apparent as well. These include secondary neoplasms such as acute myeloid leukemia (AML; e.g., associated with etoposide treatment) and radiotherapy-associated brain tumors, cardiac late effects (anthracycline therapy-associated cardiomyopathy), and neuropsychologic (e.g., methotrexate therapy-associated) and endocrinologic deficits *(89,90,98–101)*.

With regard to secondary leukemias in our most recent trial, ALL-BFM 90 *(15)*, a very low rate of secondary AML (6/2178 patients) was found, at a median observation time similar to that in another contemporary treatment program *(102)*. Whether the long-term toxicity will be diminished by this new regimen, in particular with respect to the development of secondary malignancies, remains to be determined. Follow-up times are too short to permit assessment of the cumulative incidence of other secondary malignancies, in particular secondary brain tumors *(98)*. Nevertheless, ALL-BFM 90 is the first large trial for childhood ALL in which no patient subset received more than 12 Gy of preventive cranial radiotherapy and in which the cumulative incidence of CNS-related recurrences at 6 yr was only 3% *(15)*. With regard to the late effects of high-dose methotrexate, a critical comparison between patients treated with chemoprophylaxis based on intensive use of intravenous methotrexate and intrathecal methotrexate (or triple-drug intrathecal therapy) and patients treated with preventive radiotherapy, or combinations of both, is needed to decide which regimen possesses the least long-term toxicity. The strategy chosen in ALL-BFM 90, use of only 12 Gy of preventive cranial radiotherapy and limited intrathecal methotrexate chemotherapy, might offer a reasonable alternative to other approaches.

5. PERSPECTIVE: HOST GENETIC VARIABILITY

When applying the epidemiologic triangle explaining the interrelationships among host, disease, and environment in childhood ALL (Fig. 5), it becomes clear that the environment/treatment is not solely associated with the disease but that both environment/treatment and disease are associated with the host *(103)*. Therefore, host factors potentially contribute in large part to the variability in treatment outcome observed in uniformly treated specific disease entities *(66,67)* (Table 3). Until recently, host factors in relationship with childhood ALL were mostly represented by patient characteristics such as race or gender. However, with technologic advances over the past two decades, research on the identification and contribution of

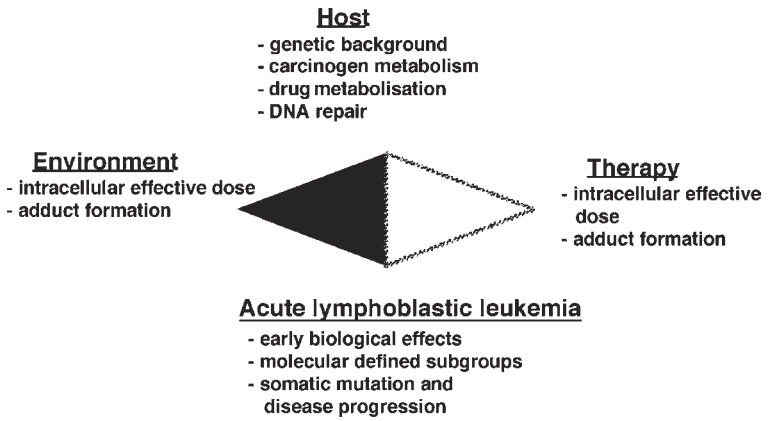

Fig. 5. Epidemiologic triangle/quadrangle *(103)* associated with childhood ALL. Interactions among host, disease, treatment, and environment are shown.

potential genetic and biochemical host factors has markedly increased. In particular, tremendous efforts within the human genome project have already resulted in a large pool of genetic information that is continously growing and will help to untangle the impact of a patient's genetic background on treatment effect and toxicity. In this context, the fields of pharmacogenetics and immunogenetics are of particular current interest to researchers working in the field of ALL.

Pharmacogenetics refers to research in which associations between genetic differences and variability in drug response are studied in defined populations *(104,105)*. The ultimate goal of pharmacogenetic studies is to develop genetic profiles for patients to optimize drug dosing, resulting in a maximum treatment effect with minimum toxicity. With regard to immunogenetics, the genetic characterization of molecular complexes such as the major histocompatibility complex (MHC), cytokines, and cellular receptors has produced a large amount of information on the role of host immunogenetic variability in disease processes *(104,105)*. These latter studies will be of particular importance for profiling susceptibility toward treatment-related infectious complications and for problems associated with, for example, bone marrow transplantation procedures. A major tool for the development of such genetic profiles are single-nucleotide polymorphisms (SNPs, pronounced "snips") *(104,105)*. SNPs describe positions within the genome where two alternative bases are observed in a population at a frequency of >1%. The latter aspect separates SNPs from simple point mutations. In addition, SNPs are stable and usually display only slight changes over several generations. From a statistical point of view, SNPs are observed in every 500–1000 bp. This frequency in the entire human genome, approx 3 billion bp, translates into an expected number of 3–6 million SNPs. It is assumed that SNPs may be responsible for as much as 90% of the genetic diversity in humans and, therefore, are suggested to play an important role in the observed phenotypic variations among individuals.

Numerous candidate genes may be of importance in childhood ALL *(105)*. With regard to treatment, most of the research on genetic variablity in patient populations conducted to date has focused on drug-metabolizing enzymes. The most extensively studied of the drug-metabolizing enzymes is thiopurine methyltransferase (TPMT), which catalyzes the S-methylation of thiopurines (e.g., 6-mercaptopurine, 6-thioguanine) *(106)*. The *TPMT* locus is subject to genetic polymorphism, with heterozygous individuals (about 10% of the Caucasian population) having intermediate TPMT activity and homozygous individuals (about 0.33% of Caucasians) having low TPMT activity *(107,108)*. More than 80% of defective TPMT activity can be explained by the most frequent variant alleles, *TPMT*3A*, *TPMT*3B*, *TPMT*3C*, and *TPMT*2*. The TPMT genotype shows excellent concordance with TPMT phenotype *(107)*. A number of clinical studies suggest that TPMT activity is associated with toxicity and outcome in childhood ALL *(106,109–114)*. A recent study at St. Jude Children's Research Hospital found the dose intensity of 6-mercaptopurine to be the single most important determinant of outcome *(113)*. Patients with lower TPMT activity fared significantly better than patients with higher TPMT activity. However, as maintenance therapy with antimetabolites is an essential element of all treatment regimens for childhood ALL, and as there is no uniform approach with regard to dose, dose-adjustment procedures, and scheduling of drugs during this treatment period, it will be important to confirm the St. Jude findings in further prospective trials and to develop strategies for implementing them in future therapeutic strategies.

Within the BFM study group, we conducted preliminary research on the genetic variability of glutathione S-transferase (GSTs) genes and their potential impact on the clinical course of childhood ALL. GSTs are a family of cytosolic enzymes involved in the detoxification of various exogenous as well as endogenous reactive species *(115)*. They function as dimers by catalyzing the conjugation of mutagenic electrophilic substrates to glutathione. In humans, four major subfamilies of GSTs can be distinguished and are designated GSTα, GSTμ, GSTθ, and GSTπ *(115)*. Each of these subfamilies is composed of several members, some of which display genetic polymorphism. Within the GSTμ subfamily, the gene coding for GSTM1 exhibits a deletion polymorphism, which in case of homozygosity (*GSTM1* null) leads to the absence of phenotypic enzyme activity *(116)*. A similar mechanism has been described for GSTT1 within the

GSTϕ subfamily, whereas the gene coding for GSTP1, a member of the GSTπ subfamily, displays polymorphisms within its coding region at codons 105 (Ile105Val) and 114 (Ala114Val) *(117,118)*. The coding region polymorphisms within *GSTP1* have been suggested to confer different catalytic activities.

In two case-control studies with BFM patients, we found protective effects of specific GST genotypes on the risk of relapse in childhood ALL *(119,120)*. The most pronounced effect was observed for the *GSTT1* null genotype. In the only other study addressing the association of GST genotypes and outcome in childhood ALL, researchers from St. Jude Children's Research Hospital reported on *GSTM1* and *GSTT1* genotypes and their impact on EFS, hematologic remission, and time to isolated CNS relapse in 161 childhood ALL patients from three consecutive trials conducted at their institution *(121)*. Except for a tendency toward higher CNS relapse-free survival at 5 yr among patients with the *GSTM1* null genotype, no particular associations between *GSTM1* and *GSTT1* genotypes, and outcome of childhood ALL were detected in that study. Although the study designs and patient characteristics in the St. Jude study were different from ours, the divergent results imply that host genetic variability may have different impacts depending on treatment characteristics.

An important focus for future studies will be the contribution of individual genotypic profiles to leukemia outcome, taking into account cytogenetic and/or molecular genetic as well as immunophenotypic features of patient populations. Certain genotypes may be associated with leukemogenesis in specific cytogenetically and/or molecular genetically defined leukemia subsets *(103,122)*. Hence, when genotypic profiles are related to leukemia outcome, it will be important to consider information associated with the etiology of the disease (see also Fig. 5).

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6 Treatment of Childhood Acute Lymphoblastic Leukemia

The Dana-Farber Cancer Institute ALL Consortium Perspective

LEWIS B. SILVERMAN AND STEPHEN E. SALLAN

CONTENTS

1. INTRODUCTION

Modern chemotherapy and supportive care have resulted in long-term event-free survival in >80% of children with acute lymphoblastic leukemia (ALL) *(1–3)*. However, therapy remains intensive, toxic, and sometimes lethal. To improve outcome for patients in the future, therapy will need to be more patient- and leukemia-specific and less toxic. Although nearly all investigators agree on this goal, there are a variety of opinions regarding how best to optimize treatment for children with ALL. In the preceding chapter (Chapter 5), Drs. Schrappe and Stanulla identified six specific areas of controversy: (1) the clinical and laboratory features that determine treatment choice, including early response to therapy; (2) the relevance of minimal residual disease measurements; (3) the intensity of initial remission induction therapy; (4) optimal central nervous system (CNS) therapy in terms of relative efficacy and toxicity; (5) optimal duration of therapy; and (6) the indications for stem cell transplantation during first complete remission. Because we are in agreement with most of the views expressed by Drs. Schrappe and Stanulla, we will focus on those areas in which there are alternative points of view. Also, we address one point not specifically considered in the previous chapter: the definition of "success," that is, whether it should be measured in terms of survival, event-free survival, or survival with the highest quality of life.

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2. CLINICAL AND LABORATORY FEATURES THAT DETERMINE TREATMENT CHOICE

Historically, each antileukemia therapeutic regimen was the same for all patients. By the mid-1970s, it had become apparent that the same therapy resulted in different outcomes; some children were cured and others were not. As therapeutic outcomes improved, investigators began to tailor treatment, reserving the most intensive therapies for those patients at highest risk of relapse. Clinical and laboratory characteristics at the time of diagnosis were identified, assigned prognostic significance as risk factors, and used to determine treatment.

Several obstacles hampered the development of a consensus on which presenting features were truly risk factors. First, every treatment group prospectively chose their own criteria, and although there was considerable overlap, there were also substantial differences, making it difficult to compare outcomes for patient subgroups treated on different protocols. Second, some important variables, such as cytogenetic findings, are unknown at the time that treatment is started, so that

initial therapy cannot always be risk-adapted. Third, as therapy improved, it became apparent that treatment itself was the single most important prognostic factor; that is, some factors, such as T-cell immunophenotype, were associated with inferior outcomes on some regimens but not on others *(1,4)*.

In the era of intensive, multiagent regimens, we are reaching the limits of prognostic significance of currently applied clinical risk factors. Results from a clinical trial we conducted between 1991 and 1995 indicated that, with the exception of age at diagnosis, the clinical and laboratory features used to risk-stratify patients were no longer prognostically significant *(1)*. On that trial, the outcomes of "high"- and "low"-risk patients were not significantly different *(1)*, suggesting that the factors used to determine risk status, although useful in directing the intensity of therapy, no longer identified those patients at highest risk of relapse. Novel prognostic factors, relating to underlying leukemia cell biology and host factors, need to be identified to improve outcome further.

As discussed by Drs. Schrappe and Stanulla, early response to initial therapy has had as significant impact on childhood ALL clinical trials, both in defining risk groups and in determining subsequent treatment *(5)*. The Berlin–Frankfurt–Münster (BFM) group convincingly demonstrated that the peripheral blood lymphoblast count after a week of monotherapy with prednisone is an important predictor of outcome *(6)*. This pivotal finding influenced investigators from the Children's Cancer Group (CCG), who subsequently demonstrated that the persistence of leukemia in bone marrow specimens obtained 7 or 14 d after the beginning of multiagent chemotherapy strongly correlated with a poor outcome *(5)*. Importantly, they have also shown that intensification of therapy could abrogate the prognostic significance of a slow early response *(7)*.

We are still in the early stages of identifying biologically relevant subsets of patients who require different curative treatments. Many investigators have reported that patients with the cryptic t(12;21) translocation (*TEL/AML1* gene fusion) have a relatively favorable prognosis, although this finding remains somewhat controversial *(8–13)*. There is emerging evidence that lymphoblasts with this rearrangement are sensitive to asparaginase therapy, so regimens that include intensive use of that agent may be especially beneficial for such patients *(14)*. Similarly, there is both in vivo and in vitro evidence that the poor outcomes for patients with *MLL* gene rearrangements (especially infants) may be improved with the use of cytarabine *(15,16)*. There is general consensus that patients with Philadelphia chromosome-positive ALL are not adequately treated with conventional chemotherapeutic regimens but have a better prognosis with allogeneic stem cell transplantation in first remission *(17)*. Such patients may also benefit from the use of STI-571, a specific inhibitor of the BCR-ABL tyrosine kinase *(18)*, although further testing is necessary to confirm the reported efficacy of this agent.

Host-related factors, such as pharmacogenetics, have not been as extensively studied as leukemia biologic factors, but they may substantially affect an individual patient's risk of relapse. Polymorphisms within genes involved in chemotherapy drug metabolism influence how rapidly and effectively a patient metabolizes certain chemotherapeutic agents. Consequently, a particular chemotherapeutic agent might be more or less effective in various patient populations based on differences in pharmacokinetics determined by host (not leukemia)-related genetics. For example, favorable outcomes have been reported in patients with mutant thiopurine methyltransferase phenotypes (involved in the metabolism of thioguanines, such as 6-mercaptopurine) *(19)* and with certain polymorphisms of the glutathione S-transferase genes (encoding enzymes involved in the intracellular detoxification of various compounds, including cyclophosphamide and corticosteroids) *(20)*. In one study, concurrent therapy with long-term anticonvulsants was associated with inferior event-free survival, perhaps owing to anticonvulsant-associated induction of drug-metabolizing enzymes (leading to increased clearance of antileukemia drugs, such as methotrexate) *(21)*. In another randomized study, conventional dosing of methotrexate based on body surface area was compared with individualizing dosing based on pharmacokinetic measurements to adjust for patient-specific clearance of this agent. Individualized dosing was associated with an improvement in outcome in children with B-lineage ALL, suggesting that some relapses on the conventional dosing arm may have been caused by rapid drug clearance *(22)*.

3. RELEVANCE OF MINIMAL RESIDUAL DISEASE MEASUREMENTS

Drs. Schrappe and Stanulla have identified minimal disease (MRD) measurements as a potentially important prognostic factor. Indeed, several investigators have reported that MRD levels early in therapy may significantly predict subsequent outcome *(23–26)*. In these studies, the risk of relapse was significantly higher in patients with detectable MRD at the end of induction therapy and early during postremission therapy *(23,24,26)*. High levels of MRD measured as early as d 15 of induction therapy have also been correlated with a poor outcome *(25)*. Of importance for clinical investigation is the finding that peripheral blood sampling may be as reliable as bone marrow sampling for the detection of MRD *(27)*.

Although MRD measurement remains a promising avenue of research, its clinical relevance in childhood ALL has not yet been fully established. It remains to be determined, for example, whether these technologically complex measurements provide additional useful prognostic information beyond that obtained by microscopic examination of peripheral blood and/or marrow after 7–14 d of induction chemotherapy. Additionally, differences in techniques (flow cytometric vs polymerase chain reaction-based analyses), measurement time points, and assay sensitivities may limit the ability to compare data from various studies. Finally, similar to previously established risk factors, the prognostic relevance of MRD is likely to be highly dependent on therapy, and so it might not be meaningful to extrapolate results between clinical trials.

4. INTENSITY OF INITIAL REMISSION INDUCTION THERAPY

When treated with a two-drug regimen of weekly vincristine and daily prednisone, nearly 90% of children with ALL will achieve remission at the end of 1 mo of therapy *(28,29)*. With

the addition of a third agent, such as asparaginase or an anthracycline, complete remissions can be induced in approximately 95% of children with ALL *(30,31)*. In addition to improving remission rates, intensified three-drug induction regimens also prolong remission duration. The importance of induction intensity in determining overall survival was demonstrated in a study conducted at the Dana-Farber Cancer Institute in which children were randomly assigned to receive identical therapy except for induction drugs; one group received vincristine and prednisone, and the other received those two drugs plus an anthracycline. Although the complete remission rates for both groups exceeded 90%, there was long-term benefit for the more intensively treated group (event-free survival rates for the two groups at 16 yr were 37 and 63%, respectively) *(31,32)*.

In theory, intensified induction regimens may prevent the new emergence of drug-resistant leukemic clones by producing an initial leukemic cell lysis of greater rapidity and magnitude *(33)*. Thus, many groups have attempted to improve long-term event-free survival through intensification of early therapy. In terms of long-term survival, the benefit of utilizing four drugs during induction therapy (vincristine, prednisone, asparaginase, and an anthracycline) is widely accepted in higher-risk patients *(34)* but less so in lower-risk patients *(35)*. Among the studies with the best reported outcomes, most rely on at least four drugs for remission induction for all patients, regardless of their risk-group status *(3,6,36)*. Since 1981, we have utilized a five- or six-agent remission induction regimen for all patients, with very favorable long-term outcomes *(36)*. Moreover, the incidence of mortality during the multiagent remission induction phase has remained approximately 1%, comparable to that reported for less intensive induction regimens *(1,2)*. As noted by Drs. Schrappe and Stanulla, the relative efficacy and toxicity of dexamethasone and prednisone during the induction phase (as in all subsequent phases of therapy as well) are under active investigation *(37)*.

5. OPTIMAL CNS THERAPY

All current treatment regimens for ALL include therapy directed at treating CNS leukemia. Historically, the most successful CNS treatment was 2400 cGy of cranial radiation (with intrathecal chemotherapy). However, because of the long-term toxicities associated with this treatment, including neurocognitive deficits, short stature, and risk of second malignancies, investigators have studied alternatives, hoping to find less toxic, equally efficacious alternatives. These alternative therapies, often used in combination, include lower doses of cranial radiation (1200–1800 cGy), frequent dosing of intrathecal chemotherapy, and CNS-directed systemic chemotherapy, such as high doses of antimetabolites.

Many investigators have eliminated cranial radiation altogether, with the goal of minimizing long-term CNS sequelae. Successful elimination of cranial radiation depends on the substitution of equally effective CNS treatment, such as high-dose systemic and/or intensive intrathecal chemotherapy. Most investigators have utilized multiple cycles of high-dose antimetabolites and/or frequent doses of intrathecal chemotherapy over prolonged periods *(38–42)*. The elimination of cranial radiation without concomitant substitution of alternative CNS-directed therapy has been associated with excessive CNS relapses in lower-risk patients *(39,43)*.

Although several studies have indicated that intensive intrathecal and systemic chemotherapy can be as effective as cranial radiation in preventing CNS relapses, especially in lower-risk patients, the relative acute and late toxicities of these CNS treatment strategies remain unsettled. A recent study of long-term survivors treated with 1800 cGy of cranial radiation on Dana-Farber Cancer Institute ALL Consortium protocols did not find any significant neuropsychological impairments in patients who were 3 yr or older at the time of diagnosis *(44)*, suggesting that lower doses of cranial radiation were not as toxic as 2400 cGy. Moreover, there is evidence that cognitive deficits are present in long-term survivors treated without cranial radiation *(45)*, and in one study, there were no significant differences in the severity and range of deficits between irradiated and nonirradiated patients *(46)*. Additionally, higher rates of acute neurotoxicity, including seizures, have been observed in patient treated with regimens that included high-dose methotrexate and intensive intrathecal chemotherapy *(47)*.

It needs to be recognized that all CNS-directed therapy, by intent, affects the brain and therefore is likely to be associated with short- and/or long-term neurotoxicity. Additionally, other systemic agents, not specifically directed toward the CNS, may have CNS toxicities. For instance, the use of dexamethasone instead of prednisone during postremission therapy (without any change in CNS-directed therapy) may be associated with more severe neurocognitive late effects *(48)*. To determine optimal CNS treatment truly, it is imperative that investigators focus on the long-term neurologic sequelae of their therapeutic regimens. Thus, until the relative efficacy and toxicity of these various approaches are better delineated, the optimal CNS treatment in childhood ALL will remain uncertain.

6. OPTIMAL DURATION OF THERAPY

The optimal duration of therapy remains unknown. Most investigators continue to treat patients for 2–3 yr, based on results of older studies in which patients received therapy that was less intensive than current regimens *(49)*. Some early studies suggested that the optimal duration of therapy may be different for boys and girls, with boys benefiting from a more prolonged continuation phase *(50)*, although this finding may be less relevant with more intensive regimens.

Even with intensive regimens, attempts to shorten therapy duration from 2 yr have not been successful. As summarized by Drs. Schrappe and Stanulla, the BFM group randomized patients to receive 18 or 24 mo of treatment and observed a higher relapse rate in patients who received the shorter treatment *(6,51)*. Similarly, high relapse rates were observed in a nonrandomized study conducted by the Tokyo Children's Cancer Study Group, in which patients received intensified therapy for only 12 mo, suggesting that truncated therapy, even if intensive, was inadequate for most children with ALL *(52)*.

Ongoing studies of MRD (discussed above) may help to clarify the optimal therapy duration for patients. In addition to quantitative levels of residual disease, differences in the proliferative and growth potentials of the remaining leukemia cells are important considerations in determining optimal duration

of therapy. For example, clinical trials in patients with rapidly proliferating, mature B-cell ALL have shown that short, intensive regimens (lasting only a few months) are quite effective *(53,54)*. On the other hand, disease with cells with a lower proliferative activity, such as some B-progenitor ALL, might benefit from a longer course of therapy.

7. STEM CELL TRANSPLANTATION DURING FIRST COMPLETE REMISSION

Because most children with newly diagnosed ALL will be cured with chemotherapy, the use of stem cell transplantation (SCT) in first remission is reserved for patient subsets with poor prognoses, such as those with the Philadelphia chromosome, as well as those with initial induction failure. Preliminary studies have suggested that some patients with Philadelphia chromosome-positive ALL, including those with low presenting leukocyte counts or favorable response to a prednisone prophase, may be successfully treated with intensive chemotherapy *(55,56)*. However, most investigators (ourselves included) recommend that all patients with Philadelphia chromosome-positive ALL be treated with an allogeneic SCT in first remission. Indeed, the report that included the largest number of patients (267 children with Philadelphia chromosome-positive ALL treated by 10 study groups between 1986 and 1996) indicated that transplantation in first remission from an HLA-matched related donor was superior to intensive chemotherapy alone for these patients *(17)*.

There is some controversy regarding the use of allogeneic SCT in first remission for patients with an *MLL* gene rearrangement, especially infants. Some investigators recommend SCT based on the poor outcome of these patients with chemotherapy *(57)*. However, there are few published data to indicate that SCT effectively treats these patients.

8. DEFINITION OF SUCCESS

Over the past 20 years, the overall 5-yr event-free survival rate for children with ALL has improved from 40% to >80%, and overall survival rates approach 90%. Unfortunately, the cost of cure must be measured in the context of morbidity for most survivors. Organ systems adversely affected by leukemia and its treatment include the brain, heart, and bone. Thus, the quality of long-term survival may be compromised by late effects of the disease and its treatment.

How, then, should "success" be measured when evaluating the results of clinical trials in childhood ALL? Historically, for the clinical investigator, *event-free survival* has been considered the gold standard because it reflects antileukemia efficacy and treatment-related mortality and provides an accurate early indicator of survival outcome in almost all series. For patients, *overall survival* is the ultimate objective. Consideration of quality of life is an important component of any definition of successful treatment.

As modern therapies reduce the risk of relapse, there is a consequent increasingly important need to address issues of treatment intensity and late sequelae. Considering event-free survival alone does not take into account the number of patients who are ultimately cured or the costs of that cure. To make rational therapeutic decisions in the future, the risks and effects of primary and salvage therapies and their impact on overall survival must be better understood with the ultimate goal of improving survival and minimizing toxicity. Measurements of quality-adjusted overall survival, such as the QTWiST method (quality-adjusted time without symptoms of disease or toxicity of treatment) *(58)*, take into account both the quantity and quality of lives saved and thus may ultimately become the gold standard when measuring the success of treatment regimens.

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CHEMOTHERAPEUTIC STRATEGIES

II

C

ACUTE LYMPHOBLASTIC LEUKEMIA
IN ADOLESCENTS AND YOUNG ADULTS

7 Treatment of Acute Lymphoblastic Leukemia in Adolescents and Young Adults

Lessons Learned and Future Directions

SIMA JEHA AND HAGOP KANTARJIAN

CONTENTS

1. INTRODUCTION

Acute lymphoblastic leukemia (ALL) has a bimodal age distribution with an early peak between 2 and 6 yr of age and a second peak in the fifth decade. The yearly incidence of ALL in the United States is about 3000 new childhood cases and 1500–2000 new adult cases. Although 70–80% of children with ALL are cured with current therapies, the results in adult ALL remain modest, despite implementation of treatment strategies that have proved successful in children. In pediatric and adult trials, age remains a major prognostic factor: outcome gradually worsens with increasing age. Adolescents and young adults (aged 12–20 yr) with ALL do worse than children but better than older adults. Younger adults are arbitrarily treated on pediatric or adult protocols, which confounds analyses of treatment outcome in this age group. In this chapter we discuss the treatment results for young adults with ALL on childhood and adult protocols, with their particular characteristics, and we propose future therapeutic plans.

2. AGE AS A PROGNOSTIC FACTOR

Prognosis in ALL has been associated with host and disease characteristics, including age, performance status, organ function, drug metabolism, leukemic cell biology, degree of leukocytosis, karyotype, immunophenotype, and time to achieve a response. Age is one of the most powerful predictors of treatment outcome in both pediatric and adult studies *(1–4)*. The increased incidence of unfavorable ALL subtypes with increasing age contributes to the worse outcome of older patients. In an Italian multicenter retrospective analysis, Baccarani et al. *(5)* reviewed the outcome of 293 adolescent and adults with ALL. Age was negatively associated with complete remission (CR) rate and duration. Adolescents (aged 11–15 yr) had the highest remission rate (91%), which did not differ significantly from that among young adults (aged 16–29 yr). In contrast, the CR rate of adults (aged 30–59 yr) and the elderly (aged 60 yr or older) was <70%. The best cutoff point to demonstrate the relationship of age to CR rate was around the age of 30. Relapse rates were lower in adolescents, intermediate in young adults, and highest in older adults. Treatment was variable in this cohort; hence, the effect of age vs therapy on patient prognosis needs evaluation in patients receiving similar regimens.

2.1. Outcome of ALL Regimens Used Across Age Groups

In a cooperative prospective study conducted in Argentina *(6)*, 390 children (younger than 16 yr) and 75 adults with ALL treated on similar regimens had remission rates of 84 and 61%, respectively. Median survival was 10 mo for adults, 12 mo for

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Table 1
Outcomes of Different Treatment Programs by Age[a]

Author	Regimen	Median age (range) (yr)	No. of patients	CR (%)	Induction mortality (%)	Survival		
						Median (mo)	3-yr (%)	5-yr (%)
Gee et al. *(7)*	L2	23 (15–78)	23	78	—	25	—	25
		4 (0.6–15)	75	98	—	54	—	70
Schauer et al. *(8)*	L10	28 (15–73)	34	85	9	51	60	45
	L10-M	24 (16–65)	39	84	5	51	60	45
Hussein et al. *(10)*	SWOG-L10	28 (15–85)	168	68	17	—	—	28
		15–19	33	91	—	43	—	45
		20–29	56	84	7	21	—	15
		30–49	39	62	—	—	—	15
		>50	40	35	50	1.1	—	10
Gaynor et al. *(9)*	L2–L17M	—	199	82	9.5	—	—	40
		<25	94	88	—	—	—	45
		25–50	73	82	—	—	—	40
		>50	32	62	—	—	—	0
Hoelzer et al. *(11)*	BFM	25 (15–65)	368	74	11	24	—	37
		<35	—	77	—	31	—	43
		>35	—	66	—	15	—	21
Larson et al. *(13)*	CALGB	32 (16–80)	197	85	—	—	—	—
		<30	—	94	1	—	69	—
		30–59	—	85	8	—	39	—
		>60	—	39	50	—	17	—
Kantarjian et al. *15*	Hyper-CVAD	39.5 (16–79)	204	91	6	—	—	39
		<30	—	98	—	—	—	54
		30–59	—	90	—	—	—	48
		> 60	45	79	16	—	—	25
Gaynon et al. *(16)*	CCG	<1	135	—	—	—	—	38
		1–9	3879	—	—	—	—	79
		≥10	1107	—	—	—	—	66
Maloney et al. *17*	POG	1–10	1550	—	—	—	—	74
		>10	345	—	—	—	—	57
Pui et al. *(18)*	Total13A	1–10	117	—	—	—	—	87
		>10	43	—	—	—	—	58
Schrappe et al. *(19)*	BFM	1–10	1733	—	—	—	—	82
		>10	386	—	—	—	—	64

Abbreviations: SWOG, Southwest Oncology Group; BFM, Berlin–Frankfurt–Münster; CALGB, Cancer and Leukemia Group B; CCG, Children's Cancer Group; POG, Pediatric Oncology Group; HyperCVAD, Hyperfractionated cyclophosphamide, vincristine, Adrianycin, and dexamethasone.
[a]Dashes indicate unavailable data.

high-risk children, and 26 mo for standard-risk children. Worse remission rates, remission durations, and survivals were also reported in adults compared with children treated with a similar intensive regimen, the Memorial Sloan-Kettering Cancer Center (MSKCC) L-2 protocol *(7)*. The incidence of complete remission was 78% in 23 adults (older than 15 yr) and 98% in 75 children younger than 15 yr. The 5-yr survival rates were 25 and 70%, respectively. In recent studies, including the continuation of the MSKCC protocols, adolescents have been treated on pediatric or adult trials. This makes outcome comparison difficult because of variability among study groups and inclusion criteria. Still, older patients have a consistently poorer outcome in both pediatric and adult trials (Table 1).

2.2. Adolescents and Young Adults on Adult Leukemia Protocols

The effectiveness of the L-2 protocol suggested that additional initial cytoreduction followed by multiagent intensification regimens may offer adults with ALL the greatest likelihood of achieving long-term disease-free survival. The L-10, L-10M, L-17, and L-17M protocols used at MSKCC to treat ALL patients older than 15 yr showed that age persisted as a major prognostic factor *(8,9)*. The CR rate decreased from 88% in patients younger than 25 yr to 62% in patients older than 50 yr. The L-10M protocol yielded inferior results in the cooperative Southwest Oncology Group (SWOG) trial, compared with those obtained at MSKCC *(10)*, because of the older population enrolled (24% of patients were older than 50 yr compared

with 10% in the MSKCC study) and the inclusion of patients with Philadelphia chromosome (Ph)-positive ALL. A Cox regression analysis of survival in the SWOG study showed age to be the only significant prognostic factor for complete remission, survival, relapse-free survival, and remission duration. Patients younger than 20 yr had a 91% CR rate and a median survival of 43 mo, whereas patients older than 50 yr had a CR rate of 35% and a median survival of 1 mo.

In a prospective multicenter German study *(11)*, CR and continuous CR (CCR) rates at 5 yr were 74 and 37%. The median age was 25 yr, patients older than 65 yr were excluded. Patients older than 35 yr had a worse outcome (CCR 21% vs 43% for younger patients). The Cancer and Leukemia Group B (CALGB) demonstrated the adverse effect of age on outcome in several of their studies. In study 8011 *(12)*, patients younger than 30 yr responded more frequently and had a better CR duration and survival. Fifty-three percent of patients younger than 20 yr remained in CR compared with 25% of patients aged 20–40 yr and 18% older than 40 yr. Only 1 of 43 patients older than 60 yr remained in CR. No difference in CR duration was observed between patients aged 20–39 and those aged 40–59. In the subsequent 8811 study, cyclophosphamide was added to the induction regimen. Consolidation was a modified BFM program with increasing doses of cyclophosphamide and the addition of 2 wk of vincristine and asparaginase treatment during the period of myelosuppression *(13)*. CR rate was 94% in patients younger than 30 yr, but only 39% in those older than 60 yr. Estimated survival at 3 yr was 69% in patients younger than 30 yr, 39% in those aged 30–59 yr, and 17% in patients older than 60 yr (Table 1). A similar five-drug induction program (CCG-192P) yielded a CR rate of 96% in children with high-risk ALL *(14)*. The M.D. Anderson Cancer Center (MDACC) reported that with the dose-intensive hyperfractionated cyclophosphamide, vincristine, adriamycin, and dexamethasone (hyper-CVAD) regimen, there was only a slight trend toward an association between the degree of leukocytosis and survival, a phenomenon not observed in previous studies of childhood or adult ALL. However, as in the preceding VAD study by this group, age had significant prognostic impact. Patients younger than 30 yr had a CR rate of 98% and an estimated 5-yr survival of 54%, compared with 79 and 25%, respectively, for patients older than 60 *(15)*.

2.3. Adolescents on Pediatric Leukemia Protocols

Within the pediatric age group, age has a significant influence on prognosis. Infants and adolescents have a worse prognosis than children of intermediate age *(16–19)*. In a retrospective Pediatric Oncology Group (POG) analysis, adolescents with ALL had a slow response to therapy and a significantly worse disease-free survival than children aged 1.5–10 yr. Adolescents were also more likely to have high leukocyte counts and unfavorable immunophenotypes and karyotypes. Numerous pediatric studies have confirmed that adolescents (11–15 yr old) have a worse prognosis than children 1–9 yr of age (Table 2) *(20–22)*. However, the variability among risk groups and therapy makes comparison of results from different clinical trials difficult.

Table 2
Four-Year EFS (%) for Children with B-Precursor ALL Treated in POG (ALINC-14) Study and CCG (100 and 1800 Series) Clinical Trials

	Age (yr)			
WBC × 10^9/L	*1–2*	*3–5*	*6–9*	*>10*
<10	82.9	84.7	82.0	69.6
10–49	74.6	74.5	80.2	59.2
>50	68.3	73.9	47.5	41.1

Abbreviations: CCG, Children's Cancer Group; EFS, event-free survival; POG, Pediatric Oncology Goup; WBC, white blood cells.

Table 3
Five-Year EFS of Patients Receiving Standard Therapy or Augmented Therapy According to Age and WBC at Diagnosis

		5-Yr EFS (%)	
WBC × 10^9/L	*Age (yr)*	*Standard Therapy*	*Augmented Therapy*
>50	1–9	42	85
>50	>9	48	67
<50	>9	66	73

Abbreviations: *see* Table 2 footnote.

In 1993 the Cancer Therapy Evaluation (CTEP) and National Cancer Institute (NCI) sponsored a workshop that included representatives from the Children's Cancer Group (CCG), POG, Dana-Farber Cancer Institute, St. Jude Children's Research Hospital, and CTEP *(23)*. A review of pediatric studies confirmed that worse prognosis was associated with leukocytosis and older age (Table 2). Uniform criteria for risk-based treatment assignment of children with ALL were suggested. Standard-risk ALL, associated with a 4-yr event-free survival (EFS) of 80%, was clinically defined by age 1–9 yr and a leukocyte count <50 × 10^9/L at diagnosis. Other presentations were considered high risk with a 4-yr EFS of 65%. It was agreed that risk groups may be refined by prognostic factors other than age and leukocyte count, such as the specific biologic properties of leukemic cells and early response to treatment. Using the above risk categories, the CCG randomized children with high-risk ALL to standard vs augmented Berlin–Frankfurt–Münster (BFM) therapy *(24)*. In all subgroups analyzed, augmented therapy was associated with an improved 5-yr EFS. The difference was significant in patients aged 1–9 yr (42% vs. 85%). Among patients older than 9 yr with a leukocyte count <50 × 10^9/L, there was little benefit from augmented therapy (EFS 66% vs. 73%). However, those with leukocyte count >50 × 10^9/L had a significant benefit (EFS 48% vs. 67%). These results indicate that within the high-risk ALL subgroup, augmented BFM therapy was more beneficial to patients with high leukocyte counts than to those who were older (Table 3), possibly because of the marked improvement in outcome of T-lineage ALL with use of augmented therapy.

3. BIOLOGIC CHARACTERISTICS

ALL is fatal when untreated, and therapy is the single most important prognostic factor for cure. The biologic heterogeneity of ALL has recently been translated into disease-specific programs directed toward heterogeneous subsets of patients. Biologic features of prognostic significance have helped define risk-adapted therapy.

3.1. Morphology

L1 morphology is observed in 85% of childhood ALL cases, whereas L2 morphology predominates in adult ALL (60% of cases) *(25)*. With modern ALL regimens, the prognostic effect of L1 vs L2 morphology has disappeared.

3.2. Immunophenotype

Immunophenotype remains important for the selection of ALL therapy. Children and adults with T-cell or mature B-cell ALL, who formerly had poor outcomes, now have a favorable prognosis with specific therapies. This demonstrates the variable prognostic importance of certain features with different therapies. T-cell ALL peaks in the adolescent–young adult age group and accounts for approx 25% of adult ALL cases. Prognosis in T-cell ALL was once dismal but was substantially improved in recent trials that included high doses of cyclophosphamide, cytarabine, and asparaginase. In the L2–L17, hyper-CVAD, and BFM studies, in which cyclophosphamide and cytarabine pulses were used, a mature T-cell immunophenotype emerged as a favorable prognostic feature by multivariate analyses *(9,11,15)*. The prognosis of immature T-cell phenotype is debatable and may still be unfavorable. The outcome of mature B-cell ALL was also poor with conventional ALL therapies. The use of hyperfractionated cyclophosphamide alternating with high doses of methotrexate and cytarabine has improved results in children and adults with B-cell ALL *(26,27)*. Improvement was most marked in patients younger than 60 yr of age. With hyper-CVAD, the 3-yr survival was 77% in patients younger than 60 yr compared with 17% for older patients *(28)*. Similar results were obtained by Hoelzer and Gale *(29)* in the German trials.

The favorable common ALL phenotype occurs in 75% of childhood ALL cases, but in only 50% of adult ALL. Phenotypically undifferentiated (or null-cell) ALL, an unfavorable subtype, occurs in 20% of adults compared with 10% of childhood ALL cases. Currently, T-cell ALL has the best prognosis, common ALL an intermediate prognosis, and null-cell ALL a slightly inferior prognosis *(29)*. Leukocyte count, age, time to CR, and immunologic subtype are four independent prognostic factors for disease free survival (DFS) by multivariate analysis *(11)*. Myeloid-positive markers have no prognostic implication in ALL *(30)*.

3.3. Cytogenetic-Molecular Studies

Karyotype at diagnosis is an important prognostic factor in both childhood and adult ALL *(31–33)*. The incidence of recurrent cytogenetic-molecular abnormalities is very different in childhood vs adult ALL. Two favorable abnormalities, hyperdiploidy > 50 and t(12;21), occur mainly in children 1–9 yr old and are rare in the adult population *(34–37)*. Trisomy of chromosomes 4 and 10 has been associated with a very low risk of treatment failure in children (4-yr EFS, 97%). In adult ALL, hyperdiploidy > 50 is infrequent (< 5% compared with 28% in children) *(37,38)* and is often associated with the Philadelphia chromosome (Ph) abnormality, t(9;22). The high incidence of t(9;22) in this ploidy group accounts for the inferior outcome of hyperdiploidy in adult ALL, since patients with hyperdiploidy > 50 without the Ph have a favorable outcome (3-yr EFS, 52%) *(31)*.

Over half of adults with ALL have pseudodiploidy. Ph-positive ALL is the most frequent abnormality in this age group (20–30% vs < 5% in children); it increases with age, peaking at around 40–50 yr *(39,40)*. Children with t(9;22)-positive ALL are more likely to be older and to have a higher incidence of leukocytosis and CNS leukemia. The classic t(9;22) is associated with a dismal prognosis in both pediatric and adult ALL *(41)*. Prolonged DFS has been observed in children with Ph variants compared with the classic t(9;22)(q34;q11) *(42,43)*. Children with Ph-positive ALL and low leukocyte counts at diagnosis, or a good early response to prednisone therapy, may be cured by intensive chemotherapy alone (5-yr EFS, 55%) *(44,45)*. Poor responders to prednisone are older and have a higher leukocyte count and a <15% chance of prolonged remission *(45)*. In a multicenter analysis of children with Ph-positive ALL, the 5-yr EFS estimate was inferior in children 10–20 yr old (<20%) compared with younger patients (>30%). Children younger than 10 yr with a leukocyte count $< 50 \times 10^9$/L had the best prognosis (DFS, 48%) *(46)*.

MLL gene rearrangement, caused by translocations involving 11q23, is another genetic abnormality associated with poor prognosis *(31,47,48)*. The most common translocation involving11q23, t(4;11)(q21;q23), occurs in 70–80% of children younger than 1 yr old and accounts for the dismal outcome in this age group. Infants lacking this rearrangement have an outcome comparable to that of older children *(49,50)*. In all age groups this translocation is associated with hyperleukocytosis, a CD10-negative early pre-B or pre-B phenotype (often with coexpression of myeloid-associated antigens) and a poor prognosis. Adults with this translocation have a shorter EFS than do children *(51)*. Children older than 1 yr have a 2% incidence of 11q23 abnormalities compared with 6% incidence in adults *(52–54)*. Patients with 11q23 rearrangements not caused by t(4;11) had the same poor outcome as did patients with t(4;11), although they did not present with the high-risk factors usually associated with t(4;11), suggesting that prognosis might be determined by the adverse effects of the 11q23 breakpoint rather than by other risk factors.

As with the B- and T-cell phenotypes, t(1;19), which occurs in 3% of young adults, has lost its poor prognostic impact with wider use of intensified protocols *(31)*.

4. PHARMACOKINETIC CONSIDERATIONS INFLUENCING AGE-RELATED PROGNOSIS

Chemotherapy selection, dosage schedule, route of administration, and drug metabolism all influence the probability of cure in ALL. Methotrexate given intravenously at intermediate-high doses, compared with standard oral doses, reduces the incidence of testicular relapse by 10-folds *(55)*. Given intrathecally and in intermediate-high dosages intravenously,

methotrexate and cytarabine reduce the frequency of meningeal relapse to <5% *(56)*, alleviating the need for prophylactic craniospinal irradiation. Fractionated high-dose cyclophosphamide and high doses of methotrexate and cytarabine dramatically improved prognosis in mature B-cell ALL, increasing the cure rate from 10% to >60% *(57–59)*. Limiting the vincristine dose to 2 mg may result in subtherapeutic levels in patients with a large body surface area. Some patients fail treatment because of inadequate doses of drugs resulting from individual variations in clearance *(60)*. It may be possible to improve existing therapeutic regimens further by adjusting dosages on the basis of individual pharmacokinetic characteristics *(60)*.

Phenotype and genotype play an important role in the selection of treatment. T-lymphoid cells may be more susceptible to cytarabine because of their high capacity for its phosphorylation to the active intracellular metabolite and low capacity for its dephosphorylation. Individuals vary in their processing of antimetabolites such as methotrexate and 6-mercaptopurine. When antimetabolites are the prime therapy in B-precursor ALL, chromosome number appears to transcend all other factors for curability *(61)*. The favorable prognosis for children with hyperdiploid B-precursor ALL treated with antimetabolite-based therapy may be explained by the high capacity of hyperdiploid common lymphoid leukemia cells for polyglutamation of methotrexate. Increased copies of chromosome 21 in B-precursor ALL blasts are generally associated with increased expression of the reduced folate carrier (*RFC*) gene, which is predictive of methotrexate uptake *(62)*. Polyglutamated forms of methotrexate are similar to methotrexate in their ability to inhibit dihydrofolate reductase. However, the polyglutamates are retained within the cell longer than the parent drug and are potent inhibitors of several enzymes in *de novo* purine synthesis that are not inhibited by methotrexate *(63,64)*. Increased formation of long-chain methotrexate polyglutamates in vitro correlated with better prognosis in pediatric B-lineage leukemias *(65)*. B-lineage blasts in adult ALL accumulate significantly lower levels of methotrexate polyglutamates than comparable cells in childhood ALL *(66)*. This may be one of many examples of differences in age-related drug metabolism that might explain variations in prognosis with similar drug regimens.

Another potential explanation for the worse outcome in older patients with ALL may be related to expression of the multidrug resistance (MDR1)-associated membrane protein (p170). MDR1 functions as a membrane adenosine triphosphate-dependent efflux pump whose increased expression results in resistance to anthracyclines, epipodophyllotoxins, vinca alkaloids, and some alkylating agents *(67,68)*. Expression of MDR1 at diagnosis had no effect on the probability of achieving CR in childhood ALL, whereas in adult ALL the CR rate was significantly lower in MDR-positive cases (56%) compared with MDR-negative cases (93%). MDR-positive patients were significantly more likely to relapse in both age groups (100% of adults and 73% of children) *(69)*. Adult ALL cells are more resistant to prednisolone, L-asparaginase, and vincristine than are pediatric ALL cells *(70)*, which is reflected by the worse response of adults to induction therapy.

5. ROLE OF HOST TOLERANCE AND COMPLIANCE

An important factor in determining outcome is the patient's ability to tolerate treatment. Organ functions deteriorate with age, resulting in age-related differences in the metabolism of chemotherapeutic agents. Older patients have poor marrow reserve and increased extramedullary toxicity, making them prone to life-threatening infections, organ failure, treatment delays, and reductions in chemotherapy dose. Induction deaths increase significantly with age, the risk of early death being lowest in patients younger than 30 yr *(5,13)*. This risk increases progressively, with most early deaths occurring in patients older than 60 yr *(10,12,13,15,28)*. The high incidences of toxicity and early mortality contribute to the poor outcome in older patients and frequently necessitate dose reductions *(13,15,28)*. Adults receiving less than the specified chemotherapy dose-intensity treatment fare substantially worse than those receiving the full treatment *(71)*. The lower frequency of adults who receive full doses of cytostatic drugs within the scheduled treatment time may contribute to the lower CR rates and briefer remissions. Organ function is better preserved in pediatric patients, who in turn show better toleration of treatment. Children are more likely to be treated at large tertiary centers and to be followed more closely by their primary physicians. Better DFS rates have been reported among patients aged 15–20 yr who have been treated in pediatric departments than those of similar ages treated by community internists *(40,72)*. Finally, parents of children with cancer may be more compliant with intensive, prolonged chemotherapeutic regimens than adult patients.

6. PITFALLS IN TREATMENT EVALUATION

As discussed earlier, prognosis in ALL depends on a number of variables related to disease biology, type and intensity of treatment, and host factors such as age, performance, and organ function. Different regimens must be studied not only for their influence on outcome but also for other variables that could influence response. Age has a great impact on ALL outcome because of differences in disease biology and host tolerance in younger vs older patients. The change in prognosis with increasing age is probably continuous and gradual. In ALL clinical trials, adolescents and young adults are usually assigned arbitrarily to childhood vs adult studies, making analysis of their outcome difficult, as comparisons involve not only treatment regimens but study group differences. Some reports estimate that about 40% of adults with ALL are cured. This figure may be an overestimate because of patient exclusion criteria and the use of DFS as an end point, which considers only patients who achieve CR and excludes 10–30% of individuals who fail to enter remission. Differences in treatment results are usually attributed to treatment modifications but are sometimes related to different study group characteristics. Some adult ALL studies include patients older than 12 yr; others include only those 20 yr and older.

Linker et al. *(73)* reported superior results with a regimen consisting of a four-drug induction followed by intensive alternating cycles of non-cross-resistant chemotherapy and prolonged oral maintenance therapy. Age had no significant effect on remission duration, despite a trend for patients older than 40

to do worse, with a projected CCR of 26% vs 44% in younger patients *(74)*. Their study excluded patients older than 50 yr, and the median age of the study group was only 24 yr (range 16–48 yr). In all other studies, age was a consistent prognostic factor, significantly affecting all indicators of prognosis (complete remission, survival, relapse-free survival, remission duration; Table 1). Even within identical genetic ALL subtypes, adults had a worse survival experience than children.

The L-10 regimen resulted in different outcomes when used at MSKCC *(8)*, SWOG *(10)*, or the University of Iowa *(75)*, demonstrating the difficulty in comparing clinical trial results among centers with heterogeneous criteria for patient inclusion and data analysis. A report comparing the outcome of patients 16–21 yr of age treated on the CALGB and CCG regimens showed superior results for patients treated on the pediatric cooperative group protocol (CR rates 96% vs 93%, 6-yr EFS rate 64% vs 38%) *(76)*. Minor differences between the two regimens, as well as differences in practice and compliance between pediatricians and internists, may have contributed to the discrepancy in results. However, the observed differences were more likely owing to differences between the study groups and to latent variables not evaluated in the analysis. For example, age distribution, which was not described, might have contributed to the observed difference, as pediatric studies tend to be shifted toward the 16-yr-old age group, whereas adult studies will accrue most of the 20-yr-old patients. The CALGB study also had twice the incidence of adverse cytogenetics than the CCG study (10% vs 5%), further emphasizing the pitfalls in comparing the efficacy of two regimens when the study groups are heterogenous.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Although the therapeutic strategies that have proved effective in children have improved results in adults with ALL as well, it is clear that older age remains an unfavorable prognostic feature *(5,7,40,77,78)*. With programs similar to those use in childhood ALL, the CR rate in adults ranges from 65 to 85% and cure rates from 20 to 35%. The ability to intensify treatment further and to optimize dose schedules may be limited. Adult ALL is biologically different from childhood ALL in many ways. Features signaling a high risk for systemic relapse (e.g., older age, high leukocyte count at diagnosis, unfavorable cytogenetics, and longer times to achieve remission) are found in 60–70% of patients with adult ALL. These patients have potential cure rates of 20–25%, compared with 60–70% in low-risk patients. Adults do not tolerate chemotherapy as well as children. Thus, both leukemic cell- and host-related characteristics contribute to the worse prognosis in adult compared with childhood ALL. Adolescents and young adults have a similar disease biology and tolerance to therapy *(47,79,80)*. Multivariate analysis of prognostic factors can define different prognostic groups for whom risk-oriented therapies are justified. The prognostic importance of ALL clinical features depends on the biologic features. Progress has been made in modifying treatment according to the leukemia subtype and in providing aggressive supportive care when intensive therapy is needed. Specific modifications are required (1) for mature B-cell ALL because of its unique sensitivity to high-dose fractionated therapy; (2) for *MLL* rearrangement and Ph-positive ALL because of their resistance to standard chemotherapy alone; (3) for hyperdiploid trisomies 4 and 10 because of their excellent prognosis; and (4) for elderly patients because of their poor tolerance to treatment. Improvement in outcome and reduction in toxicity can be enhanced with the use of new agents with selective or targeted effects. Arabinosylguanine has shown considerable therapeutic promise, including CR induction in 44% of adults and children with refractory T-cell ALL *(81)*. Recent insights into the mechanisms by which tumor-specific cytolytic cells are produced have encouraged the development of targeted immunotherapy *(82)*. Monoclonal antibodies have established their role and are now used in combination with chemotherapy. The recent finding that ALL is angiogenic suggests that antiangiogenesis agents may have a therapeutic role *(83)*. The molecular genetics of acute leukemia may eventually be the most important feature in selecting treatment. The activity of a tyrosine kinase inhibitor in t(9;22)-positive ALL is an example of successful treatment of leukemia with an agent targeted to a specific molecular genetic anomaly. These strategies indicate that further improvement in ALL outcome will evolve from therapies tailored to disease biology rather than age.

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8 Treatment of Adolescents and Young Adults with Acute Lymphoblastic Leukemia

Children's Cancer Group Trials

JAMES NACHMAN

CONTENTS

1. INTRODUCTION

Adolescents with acute lymphoblastic leukemia (ALL), particularly those 16–21 yr of age at diagnosis, are an underrepresented group in both pediatric and adult clinical trials *(1)*. Thus, literature describing the treatment and outcome for such patients is limited. Moreover, because these older adolescents comprise a very small percentage of either the adult or the pediatric ALL populations, they are often combined with patients 10–15 yr of age in analyses of pediatric trials or with those 20–30 yr of age in analyses of adult trials. A further concern is the variable treatment outcome among older adolescents owing to the marked differences in clinical management strategies between pediatric and adult ALL trials. In some adult trials, for example, patients achieving an initial remission are candidates for a matched sibling donor transplant, whereas in pediatric trials, a first remission transplant is reserved for a small percentage of patients with very high-risk features.

Adolescent and adult patients with ALL have significantly worse event free-survival (EFS) and overall survival (OS) rates than do younger children. Survival data for ALL patients derived from the Surveillance, Epidemiology, and End Results (SEER) Program Registry (1986–1995) are shown in Table 1. Major decrements in survival begin to occur in patients 15–19 yr of age; further declines occur for the patients older than 30 years of age. In a report from the Children's Cancer Group (CCG), patients 16–21 yr of age with ALL treated between 1984 and 1989 had a 6-yr EFS of 59%, which was worse than the outcome observed for younger children treated in the same series of trials *(2)*. In the UKALL X and XA trials *(3)*, 5-yr EFS estimates were 62% for children 1–9 yr of age, 49% for patients 10–14 years, 35% for patients 15–19 years, and 29% for patients 20–39 years. On the Berlin–Frankfurt–Münster (BFM) 86 study *(4)*, 6-yr EFS estimates were 81% for patients 1–5 years of age, 71% for patients 5–9 years, and 65% for patients older than 10 years.

2. CLINICAL AND BIOLOGIC FEATURES OF ADOLESCENT ALL

Patients 10 or more yr of age have distinct presenting features compared with younger patients, whereas those 10–15 yr of age and 16–21 yr of age have similar presenting features. Older patients are more likely than younger patients to have a white blood cell count (WBC) of ≥ 50,000/μL, hemoglobin of ≥11 g/dL, a T-cell immunophenotype, and a t(9;22)(q11;q34) translocation [the Philadelphia chromosome (Ph); CCG, unpublished data, 1989–1995]. Numerous studies have shown that Ph+ ALL is associated with a B-lineage immunophenotype and a high WBC. For example, in a recent German-Italian study, 29 of 35 patients older than 10 yr with Ph+ ALL had an initial WBC > 50,000, and 16 had WBC >100,000 *(5)*. Adolescents with ALL also are less likely than younger patients to have high hyperdiploidy (> 50 chromosomes) and less likely to have a *TEL-AML1* translocation, both of which are favorable risk factors in ALL *(6–14)*. In CCG studies conducted between 1989 and 1995, approximately 65% of adolescents were classified as B-lineage/Ph–; approximately 6% were classified as

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Table 1
Survival Rates for Patients with ALL According to Age[a]

Age group (yr)	*5-yr survival rates (%)*
5–9	81
10–14	68
15–19	49
20–24	44
25–29	40
30–34	23

[a]SEER Program data (1986–1995).

B-lineage/Ph+; and approximately 29% were classified as T-lineage (CCG, unpublished data). In vitro drug sensitivity profiles also differ between patients younger than 10 yr of age and older patients with B-precursor ALL. Compared with their younger counterparts, patients 10 yr of age or older had a 7-fold increase in resistance to prednisone (P=0.000083), a 4-fold increase in resistance to dexamethasone (p = 0.0012), a 13-fold increase in resistance to asparaginase (p = 0.002),and a 3-fold increase in resistance to 6-mercaptopurine (p = 0.002) *(15)*.

3. TREATMENT STRATEGIES FOR ADOLESCENTS WITH ALL

Adolescents have not typically been treated as a separate risk group, rather, like other children with ALL, they were assigned to treatment based on presenting features, including age, WBC, presence of bulky disease (lymphomatous features), platelet counts, hemoglobin levels, and immunophenotype. Most adolescents were therefore assigned to protocols for higher risk patients. Prior to 1981, the EFS for patients with ALL and high risk features (>50,000 WBC/mL and lymphomatous features) treated on CCG protocols was <50%. In an attempt to improve outcome, we chose to evaluate the treatment strategy developed by the BFM Study Group, which utilized an intensive induction consolidation course and a later reinduction-reconsolidation course that substituted alternative drugs for those used with the initial induction-consolidation (dexamethasone for prednisone; doxorubicin for daunorubicin; 6-thioguanine for 6-mercaptopurine) *(16)*. We initially utilized a modified BFM-type treatment program (CCG-BFM), based on the BFM-76/79 regimen *(16)*, in a pilot trial for high-risk patients conducted between 1981 and 1983. Although subsequent BFM trials added high-dose methotrexate with leucovorin rescue as a component of presymptomatic treatment of the central nervous system (CNS) *(4,17)*, CCG did not adopt the high-dose methotrexate component of the later BFM regimens. The CCG-BFM treatment plan is shown in Table 2. Based on the excellent results of the pilot trial *(18)*, we proceeded with randomized trials to establish the superiority of CCG-BFM to previously utilized treatment regimens for high-risk patients and to evaluate the individual components of CCG-BFM for intermediate risk patients.

3.1. CCG-100 Series of Protocols (1984–1989)

In the CCG-100 series of studies, conducted between 1984 and 1989, ALL patients 10–21 yr of age were assigned to one of three treatment protocols based on presenting leukemic blast cell morphology, WBC, and presence or absence of lymphomatous features *(19)* (Table 3). The CCG-105 study *(20)* for intermediate-risk ALL treated patients with WBC < 50,000/μL and L1 blast morphology who lacked lymphomatous features and was designed to evaluate the relative contributions of the two main components of CCG-BFM therapy, intensive induction/consolidation and reinduction/reconsolidation (termed *delayed intensification*). For adolescents on this study, both intensive induction/consolidation and delayed intensification (arm A; full CCG-BFM) improved outcome compared with standard CCG therapy or either BFM component alone (arms B, C, and D). CCG-105 also compared cranial radiation therapy (CRT) and intrathecal methotrexate with intrathecal methotrexate alone for presymptomatic treatment of the CNS. Although intrathecal therapy alone was sufficient for prevention of CNS disease among younger patients, adolescents who received CRT had a better outcome than those who received intrathecal therapy alone *(20,21)*.

The CCG-106 study *(22)* for patients with high-risk ALL presenting WBC of ≥ 50,000/μL and/or French-American-British (FAB) L2 morphology and no lymphomatous feature. This study compared standard CCG therapy with CCG-BFM therapy and another aggressive program, the so-called New York (NY) therapy *(23)*, a modification of the LSA_2-L_2 regimen developed for non-Hodgkin's lymphoma *(24)*. Both CCG-BFM and NY therapy provided improved outcome compared with standard therapy. EFS and OS were similar on the two aggressive regimens for the overall group of patients, although NY therapy required more days of parenteral therapy and thus more hospitalization, as well as increased exposure to anthracyclines and alkylating agents. Approximately 37% of patients on CCG-106 were 10 yr of age or older. Among this subset, 7-yr EFS estimates were 61, 40, and 37% (p = 0.19), for the CCG-BFM, NY, and standard regimens, respectively.

The CCG-123 study treated patients with lymphomatous features, defined as the presence of bulky disease (Table 3) together with a WBC of ≥50,000/μL, a T-lineage immunophenotype, or a hemoglobin level of ≥10 g/dL. On this study, CCG-BFM therapy and NY therapy were more effective than either of two versions of the LSA_2L_2 regimen *(25)*. Six-year EFS estimates for the overall cohort were 67% on both the CCG-BFM and the NY regimens. The 10-yr EFS estimate for the overall cohort of patients 10 yr of age or older was approximately 57%.

Overall, 143 patients 16–21 yr of age were treated on the CCG-100 series of ALL trials. Among these older adolescents, 136 (95%) achieved remission, and the 6-yr EFS was 59% *(2)*. Based on the results of the individual trials, plans were adopted for future trials that assigned adolescents to the high-risk group, regardless of presenting WBC. In addition, stratification by FAB morphology was dropped, but stratification based on the presence or absence of lymphomatous features continued.

3.2. CCG-1800 Series of Protocols (1989-1995)

In the CCG-1882 trial *(26,27)*, high-risk patients were defined as those 10 yr of age or older or those with a WBC of ≥50,000/μL, excluding patients with lymphomatous features.

Table 2
CCG-BFM Therapy

Phase	*Drug*	*Dose and schedule*
Induction	PRED	60 mg/m^2 po, d 0–27
	VCR	1.5 mg/m^2 iv, d 0, 7, 14, 21
	DAUN	25 mg/m^2 iv, d 0, 7, 14, 21
	L-ASP	6000 U/m^2 im, 9 doses, d 3–21
	IT MTX[a]	d 7 and 28
Consolidation (5 wk)	PRED	Taper for 10 d
	CPM	1000 mg/m^2 iv, d 0, 14
	6-MP	60 mg/m^2 po, d 0–27
	ARA-C	75 mg/m^2/d iv, d 1–4, 8–11, 15–18, 22–25
	IT MTX a	d 1, 8, 15, 22
	Radiation[b]	A: cranial,1800 cGy
		B: cranial, 2400 cGy + spinal, 600 Gy
		C: bilateral testicular, 2400 cGy
Interim maintenance (8 wk)	6-MP	60 mg/m^2/d po, d 0-41
	MTX	15 mg/m^2/d po, days 0, 7, 14, 21, 28, 35
Delayed intensification (8 wk)	DEX	10 mg/m^2/d PO, d 0–20, then taper for 7 d
	VCR	1.5 mg/m^2 IV, d 0, 7, 14
	DOX	25 mg/m^2 IV, d 0, 7, 14
	L-ASP	6000 U/m^2 IM, d 3, 5, 7, 10, 12,14
	CPM	1000 mg/m^2 IV, d 28
	6-TG	60 mg/m^2/d po, d 28–41
	ARA-C	75 mg/m^2 sq/iv, d 29–32, 36-39
	IT MTX[a]	d 29, 36
Maintenance (12-wk cycles)[c]	VCR	1.5 mg/m^2 iv d 0, 28, 56
	PRED	40 mg/m^2/d po, d 0–4, 28–32, 56–60
	6-MP	75 mg/m^2/d po, d 0-83
	MTX	20 mg/m^2 po d 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77
	IT MTX[a]	d 0

Abbreviations: CCG-BFM, Children's Cancer Goup (CCG)-modified Berlin–Frankfurt–Münster Group (BFM) therapy; PRED, prednisone; CPM, cyclophosphamide; 6-MP, 6-mercaptopurine; VCR, vincristine; ARA-C, cytarabine; IT, intrathecal; DAUN, daunorubicin; MTX, methotrexate; DEX, dexamethasone; DOX, doxorubicin; L-ASP, L-asparaginase; 6-TG, 6-thioguanine; po, oral; iv, intravenous; sq, subcutaneous; im, intramuscular; CNS, central nervous system.

[a]Age-adjusted doses as follows: 1–1.9 yr, 8 mg; 2–2.9 yr, 10 mg; ≥3 yr, 12 mg; patients with CNS disease at diagnosis also received IT MTX on d 7 and 21 of induction and did not receive IT therapy on d 15 and 22 of consolidation.

[b]A, patients without CNS disease at diagnosis; B, patients with CNS disease at diagnosis; C, patients with testicular disease at diagnosis.

[c]Cycles of maintenance therapy were repeated until the total duration of therapy, beginning with the first interim maintenance period, reached 2 yr for girls and 3 yr for boys.

Table 3
Treatment Allocation for Adolescents with ALL on CCG Protocols (1984–1989)

Study/risk group	*Eligibility criteria*	*Treatment arms*
CCG-105/ intermediate risk	WBC< 50,000/μL; no lymphomatous features[a]	A. CCG-BFM induction/consolidation; interim maintenance and delayed intensification; standard maintenance; ± CRT B. Standard induction/consolidation; interim maintenance and delayed intensification; standard maintenance; ± CRT C. CCG-BFM induction/consolidation; standard maintenance; ± CRT D. Standard induction/consolidation; standard maintenance; ± CRT
CCG-106/ high risk	WBC ≥ 50,000/μL and/or L2 morphology; no lymphomatous features[a]	A. CCG-BFM B. New York I C. Standard therapy
CCG-123/ lymphomatous ALL[a]	Presence of bulky disease and at least one laboratory feature	A. CCG-BFM B. LSA_2L_2 with CRT C. LSA_2L_2 without CRT D. New York I

Abbreviations: CRT, cranial radiation therapy; CCG, Children's Cancer Group; BFM, Berlin–Frankfurt–Münster Group.

[a]Lymphomatous ALL was defined as the presence of bulky disease (large mediastinal mass or spleen enlarged below the umbilicus or single lymph node > 3 cm or contiguous lymph nodes > 5 cm) and the presence of WBC ≥ 50,000/μL, or T-lineage immunophenotype, or hemoglobin > 10 g/dL.

Table 4
A-BFM[i] Therapy

Phase	*Drug*	*Dose and schedule*
Induction	PRED	60 mg/m^2 po, d 0–27
	VCR	1.5 mg/m^2 iv, d 0, 7, 14, 21
	DAUN	25 mg/m^2 iv, d 0, 7, 14, 21
	L-ASP	6000 U/m^2 im, 9 doses, d 3–21
	IT MTX[a]	d 7 and 28
Consolidation (9 wk)	PRED	Taper for 10 d
	CPM	1000 mg/m^2 iv, d 0, 28
	6-MP	60 mg/m^2 po, d 0–13, 28–41
	VCR	1.5 mg/m^2 iv, d 14, 21, 42, 49
	ARA-C	75 mg/m^2/d iv or sq, d 1–4, 8–11, 29–32, 36–39
	L-ASP	6000 U/m^2 im, d 14, 16, 18, 21, 23, 25, 42, 44, 46, 49, 51, 53
	IT MTX [a]	d 1, 8, 15, 22
	Radiation[b]	A: cranial, 1800 cGy
		B: cranial, 2400 cGy + spinal, 600 Gy
		C: bilateral testicular, 2400 cGy
Interim maintenance I (8 wk)	VCR	1.5 mg/m^2/d iv, d 0, 10, 20, 30, 40
	MTX[c]	100 mg/m^2/d iv, d 0, 10, 20, 30, 40
	L-ASP	15,000 U/m^2/d im, d 1, 11, 21, 31, 41
Delayed intensification I (8 wk)	DEX	10 mg/m^2/day PO, d 0–20, then taper for 7 d
	VCR	1.5 mg/m^2 iv, d 0, 7, 14, 42, 49
	DOX	25 mg/m^2 iv, d 0, 7, 14
	L-ASP	6000 U/m^2 im, d 3, 5, 7, 10, 12, 14, 42, 44, 46, 49, 51, 53
	CPM	1000 mg/m^2 iv, d 28
	6-TG	60 mg/m^2/d po, d 28–41
	ARA-C	75 mg/m^2 sq/iv, d 29–32, 36–39
	IT MTX[b]	d 29, 36
Interim maintenance II (8 wk)	VCR	1.5 mg/m^2/d iv, d 0, 10, 20, 30, 40
	MTX[b]	100 mg/m^2/d iv, d 0, 10, 20, 30, 40
	L-ASP	15,000 U/m^2/d im, d 1, 11, 21, 31, 41
	IT MTX	d 0, 20, 40
Delayed intensification II (8 wk)	See delayed intensification I	
Maintenance (12-wk cycles)[d]	VCR	1.5 mg/m^2 iv d 0, 28, 56
	PRED	40 mg/m^2/d po, d 0–4, 28–32, 56–60
	6-MP	75 mg/m^2/d po, d 0–83
	MTX	20 mg/m^2 po d 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77
	IT MTX[a]	d 0

Abbreviations: A-BFM, augmented Berlin–Frankfurt–Münster (BFM); PRED, prednisone; CPM, cyclophosphamide; 6-MP, 6-mercaptopurine; VCR, vincristine; ARA-C, cytarabine; IT, intrathecal; MTX, methotrexate; DEX, dexamethasone; DOX, doxorubicin; DAUN, daunorubicin; L-ASP, L-asparaginase; 6-TG, 6-thioguanine; po, oral; iv, intravenous; sq, subcutaneous; im, intramuscular; CNS, central nervous system.

[a]Age-adjusted doses as follows: 1–1.9 yr, 8 mg; 2–2.9 yr, 10 mg; ≥3 yr, 12 mg; patients with CNS disease at diagnosis did not receive IT therapy on d 15 and 22 of the consolidation phase.

[b]A, patients without CNS disease at diagnosis; B, patients with CNS disease at diagnosis; C, patients with testicular disease at diagnosis.

[c]IV MTX was escalated by 50 mg/m^2 at each dose.

[d]Cycles of maintenance therapy were repeated until the total duration of therapy, beginnng with the first interim maintenance period, reached 2 yr for girls and 3 yr for boys.

Treatment allocation on this study utilized early marrow response, which had emerged as a prognostic factor in the CCG-193 pilot trial of CCG-BFM therapy *(18)*. In the CCG-193P study, patients with a slow early response (SER) to induction therapy (>25% marrow blasts at d 7) had a significantly worse EFS (<50%) compared with that of patients achieving a rapid early response (RER) to induction therapy (≤25% marrow blasts at d 7). RER patients on the CCG-1882 protocol were randomized to receive CCG-BFM therapy with either intrathecal methotrexate and CRT or intensified intrathecal methotrexate alone for CNS prophylaxis. For SER patients on CCG-1882, a new treatment program, augmented BFM (A-BFM; Table 4) was developed. The A-BFM intensification strategy was developed in response to the observation by German investigators that addition of high-dose cytarabine, high-dose methotrexate, ifosfamide, and mitoxantrone did not improve the outcome of patients who had a poor response to an initial 7-d course of prednisone *(4)*. Slow early responders enrolled on CCG-1882 were initially treated with A-BFM during a pilot feasibility phase *(28)*; subsequently they were randomized to receive CCG-BFM or A-BFM.

A-BFM was designed to increase the amount of vincristine, L-asparaginase, and steroid given during the first year of therapy and to incorporate intravenous methotrexate without leucovorin rescue into the treatment plan. Intravenous methotrexate without leucovorin rescue was utilized according to the Capizzi methotrexate regimen (vincristine and intravenous methotrexate on d 1 followed by L-asparaginase on d 2) *(29)*, which had proved effective in the treatment of relapsed ALL *(30)*. Capizzi methotrexate replaced oral 6-mercaptopurine and oral methotrexate in each of the two interim maintenance phases of therapy. Increased exposure to vincristine and L-asparaginase was obtained by incorporating 2-wk pulses of these two drugs into each consolidation and delayed intensification course. A second interim maintenance phase and a second delayed intensification phase were added prior to standard maintenance. A comparison of the total number of doses of the various drugs administered during the first year for CCG-BFM and A-BFM is shown in Table 5.

Table 5
Comparison of CCG-BFM and A-BFM Chemotherapy During the First Year of Treatment

	Number of doses	
Drug	*CCG-BFM*	*A-BFM*
Vincristine	15	30
L-Asparaginase	15	55
Cyclophosphamide	3	4
Cytarabine	24	32
Intravenous methotrexate	0	10
Dexamethasone (20-d courses)	1	2

Among RER patients, those treated with CCG-BFM, intrathecal methotrexate, and CRT had a similar outcome to those treated with CCG-BFM and intrathecal methotrexate alone, with 5-yr EFS estimates of 69 and 75%, respectively ($p = 0.50$) *(26)*. Among patients 10 yr of age or older, there were more bone marrow relapses on the CRT arm compared with the intrathecal arm (49 vs 30), suggesting that intrathecal methotrexate may have significant systemic effects. A systemic effect of intrathecal methotrexate was also suggested by results from a retrospective analysis of data from the European Organization for the Research and Treatment of Cancer (EORTC) Children's Leukemia Cooperative Group (CLCG) 58881 trial *(31)*. On CLCG-58881, patients received a week-long prednisone prophase based on that of the BFM-83 protocol *(32)*. However, on the BFM trial, intrathecal methotrexate was given on d 0, whereas on the CLCG protocol the intrathecal methotrexate was given on d 8. The frequency of poor responders to the prednisone prophase was 20% on the CLCG trial compared with 8% on the BFM trial. The CLCG trial was subsequently amended to provide intrathecal therapy on d 0, resulting in a reduction of the rate of prednisone poor response to 12%.

Among SER patients on CCG-1882, A-BFM produced a statistically significant improvement in outcome compared with CCG-BFM: 5-yr EFS of 75 and 55%, respectively ($p < 0.001$) *(27)*. Overall survival was also significantly better for patients on A-BFM compared with that of patients on CCG-BFM. These improvements were particularly evident in patients younger than 10 yr of age. For patients 10 yr of age or older, A-BFM produced a better 5-yr EFS (68%) than CCG-BFM (60%), but the result did not reach statistical significance. A significant toxicity, osteonecrosis, was observed at a relatively high frequency on CCG-1882. Nearly all cases (107 of 111; 96%) occurred in patients 10 yr of age or older; rates were similar among patients 10–15 yr of age (14%) and 16–20 yr of age (18%). Among SER patients, rates were 23% for those on A-BFM and 16% for those treated on CCG-BFM ($p = 0.27$). These differences were accentuated among females and among patients 10–15 yr of age. A detailed description of osteonecrosis will be presented elsewhere *(48)*. These data suggest that subsets of adolescents may be particularly susceptible to toxicity on A-BFM.

Among RER patients, neither regimen was superior among the 49 T-lineage patients or among the larger B-lineage subset ($n = 408$). Among SER patients, A-BFM was superior to CCG-BFM for both T-lineage ($n = 26$) and B-lineage ($n = 183$) patients. Separate analyses for the lineage groups within the adolescent subset were not done. A-BFM was particularly effective for T-lineage patients, as evidenced by their 3-yr EFS of 92%. As was the case in other CCG studies of this era, B- and T-lineage patients had similar outcomes regardless of the CCG-1882 regimen they received.

Outcome for the 23 Ph+ ALL patients treated on CCG-1882 was poor (4-yr EFS, 11%) *(33)*. A-BFM did not appear to improve outcome for SER Ph+ patients: two of three Ph+ SER patients treated with CCG-BFM and three of four SER Ph+ patients treated with A-BFM had adverse events. Both of the Ph+ patients who survived without an event received bone marrow transplants in first remission.

Adolescents with lymphomatous features were treated on the CCG-1901 study, which utilized either the intensive NY regimen described above *(25)* or a variation on that regimen, NY II, which was designed to improve efficacy while reducing toxicity of the original NY regimen. The 7-yr EFS estimate for patients 10 yr of age or older in CCG-1901 was approximately 78%. Seven of 21 patients 16–21 yr of age with lymphomatous features experienced an adverse event.

Together, 196 patients 16–21 yr of age were treated on the CCG-1882 high-risk trial ($n = 175$) and the CCG-1901 trial for lymphomatous ALL ($n = 21$). The 6-yr EFS for this combined group of older adolescents was 65.2 ± 3.5% (CCG, unpublished data). Overall results from the CCG-1901 trial, as well as its predecessor, CCG-123, indicated that patients with lymphomatous features should achieve a similar outcome to that of other high-risk patients. Thus, in our current CCG trials, patients with lymphomatous features are no longer treated as a separate risk group but are assigned to treatment based on presenting age and WBC. As a result, all adolescents with ALL are now treated as high-risk patients.

3.3. Current CCG Protocols (1996 to present)

The current CCG-1961 trial for high-risk ALL, which includes all adolescent patients, is designed to test various components of A-BFM therapy with CCG-BFM therapy. As

described earlier, the two major differences between A-BFM and CCG-BFM are (1) the increased duration of intensive therapy (inclusion of a second interim maintenance and delayed intensification phase) and (2) the use of more intensive therapy (additional vincristine and L-asparaginase in delayed intensification and Capizzi methotrexate in interim maintenance). As in CCG-1882, patients enrolled in the CCG-1961 study are stratified into RER and SER subsets for treatment allocation. RER patients are randomized to receive CCG-BFM (arm 1), CCG-BFM with a second delayed intensification phase (arm 2), A-BFM without the second interim maintenance and second delayed intensification phase (arm 3), or full A-BFM (arm 4). SER patients are randomized to receive augmented BFM with or without a new reinduction element utilizing cyclophosphamide and idarubicin. Ph+ patients are nonrandomly assigned to A-BFM with cyclophosphamide and idarubicin. To date, 247 patients 16–21 yr of age have been entered in the trial. The 1-yr EFS estimate for these patients was 88.3%, compared with 83.1% for older adolescents treated on the CCG-1882 and CCG-1901 protocols ($p = 0.06$). In an effort to decrease the incidence of osteonecrosis in this trial, all patients receiving two delayed intensification courses receive discontinuous (d 0–7 and 14–21) dexamethasone pulses. In early analyses, it appears that the incidence of osteonecrosis has been decreased as a result of the change in steroid administration.

4. DISCUSSION

The outcome of CCG therapeutic trials for adolescents and young adults with ALL has improved during successive treatment eras. Therapy remains inadequate, however, for approximately 30% of patients. The current high-risk trial of intensive therapy has shown a favorable early outcome for patients 16–21 yr of age, but longer follow-up is required for confirmation. The subset of adolescents, as well as younger children, with Ph+ ALL is of particular concern due to their very poor outcome, despite the use of intensive A-BFM therapy. A similar poor outcome for Ph+ adolescent patients was observed on retrospective analysis of two consecutive BFM (BFM-86 and BFM-91) and Italian Association for Pediatric Hematology and Oncology (AIEOP; AIEOP-88 and AIEOP-91) trials *(5)*. Among 4760 enrolled patients, 61 had Ph+ ALL. Nineteen of these were 10 yr of age or older, 17 whom received therapy on higher risk protocols. Only 5 of these 17 patients were event-free survivors, 4 of whom underwent matched related donor (MRD) bone marrow transplantation (BMT). Two of the 17 patients remained alive after relapse, with 1 receiving a mismatched related donor (MMRD) transplant. Of the 10 patients died who died, 5 had received chemotherapy, 3 an MRD transplant, and 2 an MMRD or a matched unrelated donor (MURD) transplant. Similarly, on our CCG-1882 trial, both SER Ph+ patients who were event-free survivors underwent BMT in first remission. Together, these data suggest that Ph+ patients who have a matched sibling donor should undergo BMT in first remission. The use of alternative donor transplants remains controversial. Clearly, new therapeutic approaches are needed for Ph+ ALL.

An additional area of concern for the treatment of higher risk patients is the choice of prophylactic therapy for the CNS. As described above, RER patients treated on the CCG-1882 protocol had similar EFS rates whether they received CRT and intrathecal drugs or intrathecal therapy alone, although there was a higher frequency of CNS relapse among patients who received only intrathecal therapy. SER patients in the CCG-1882 study received CRT and intrathecal therapy, as do SER patients on the current CCG-1961 high-risk trial. CCG has not investigated the use of intrathecal therapy alone for high-risk SER patients. European investigators reported a retrospective comparison of CNS prophylaxis for the subset of T-lineage patients with a good early response to induction therapy who were treated in the ALL-BFM 90 and AIEOP-ALL 91 trials *(34)*. These studies employed similar systemic therapies based on the BFM backbone, but the BFM trial used CRT and intrathecal therapy for high-risk patients, whereas the AIEOP trial relied on triple intrathecal therapy alone for CNS prophylaxis. Among patients on the BFM-90 protocol, 3-yr EFS estimates were 90% for those with a WBC <100,000/μL ($n = 99$) and 82% for those with counts of ≥100,000/μL ($n = 24$), compared with 81% ($n = 55$) and 17.9% ($n = 14$) for similar groups treated on AIEOP-91. The authors interpreted these findings to suggest that CRT was necessary for treating T-lineage patients with higher WBC values regardless of early response to treatment. These interesting data must be interpreted with caution, however, owing to the small patient numbers and the nonrandomized comparison. Overall, CNS relapse was also more frequent and the EFS rate worse for patients treated in the AIEOP trial. Some elements of systemic therapy also differed between the two trials, including the use of *Erwinia* L-asparaginase in place of *E. coli* L-asparaginase and higher doses of leucovorin rescue in the AIEOP trial.

The new generation of ALL studies will include patients from the former CCG and Pediatric Oncology Group (POG) groups. Adolescents with ALL will be will be allocated to one of the two treatment protocols based on immunophenotype (B-precursor, T). Randomized questions for the B-precursor trial will include evaluation of dexamethasone vs prednisone during induction and maintenance and the evaluation of high-dose methorexate with rescue vs Capizzi methotrexate without rescue during interim maintenance phases.

In the CCG 1922 trial for children with standard-risk ALL, the use of dexamethasone resulted in a significant improvement in EFS compared with patients receiving prednisone *(35)*. Dexamethasone-treated patients had decreased incidence of both CNS and bone marrow relapse compared with patients receiving prednisone. The CCG 1922 study utilized a three-drug induction without anthracycline. Investigators in Boston have raised concerns over a significant increase in toxicity, primarily sepsis, when dexamethasone is used as part of a four-agent induction *(36)*. A POG pilot trial of four-agent induction with dexamethasone was terminated early because of early toxic deaths owing to infection. However, Lawson et al. *(37)* utilized dexamethasone in a four-agent reinduction regimen for children and young adults with relapsed ALL and reported acceptable toxicity. In the BFM 2000 ALL trial, patients are randomized to receive dexamethasone or prednisone as part of four-agent induction following a 7-d prednisone prophase. A preliminary report shows comparable toxicity for dexamethasone and prednisone *(38)*.

High-dose methotrexate with rescue is utilized in BFM ALL regimens (protocol M) and in POG ALL trials. A head-to-head comparison of high-dose methotrexate with rescue and lower-dose intravenous methotrexate without rescue is clearly warranted.

Adolescents with T-cell ALL will receive high-dose methotrexate with rescue and will receive 1200 cGy of cranial radiation for CNS nervous system prophylaxis. Randomized questions may include dexamethasone vs prednisone during induction and maintenance and the benefit of high-dose L-asparaginase during postinduction treatment.

The T-cell-specific drug 2-amino-9-β-D-arabinosyl-6-methoxy-9H-purine (506U) has shown promise in the treatment of refractory T-lineage ALL *(39,40)*. An intergroup phase II trial of 506U in children with recurrent T-lineage ALL or non-Hodgkin's lymphoma is currently being conducted by POG and CCG. If results of this trial prove encouraging, 506U will be considered for testing in a randomized trial for patients with T-lineage ALL.

An additional area of concern with BFM-based therapy is the method of utilization of cytarabine during the consolidation and delayed intensification phases. Currently, we employ intravenous or subcutaneous cytarabine at 75 mg/m^2 at four doses/wk for 4 wk during consolidation and four doses/wk for 2 wk during delayed intensification. Pharmacologic studies of this agent suggest that prolonged exposure to therapeutic levels may be crucial for maximal cytotoxicity *(41,42)*. Additional studies have suggested that high-dose 6-mercaptopurine or L-asparaginase given before cytarabine may potentiate the cytotoxicity of the latter drug *(43,44)*. The combination of fludarabine and continuous-infusion cytarabine, with or without an anthracycline, have produced remissions in patients with relapsed ALL and acute myeloblastic leukemia *(45–47)*. Investigators at Children's Hospital of Los Angeles are currently studying a regimen of 6-thioguanine followed by continuous-infusion cytarabine for patients with relapsed ALL. Results of this trial will be useful in developing a new consolidation element based on continuous-infusion cytarabine together with a potentiating agent.

Future trials for adolescents with ALL will be conducted by the newly formed Children's Oncology Group (COG), which represents a merger of POG, CCG, the National Wilms Tumor Study Group, and the Intergroup Rhabdomyosarcoma Study Group. This merger will allow larger numbers of patients to be studied, resulting in more effective use of resources and more compelling results. Elements of the proven effective treatment strategies for higher risk ALL, together with novel treatment strategies, such as those described above, will be employed in future COG trials for high-risk ALL. COG also hopes to improve the participation of adolescents in clinical trials by implementing a number of outreach programs for adolescent patients, their families, and their caregivers. Joint efforts with the adult oncology groups should also facilitate the best standard of care for these higher-risk patients.

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CHEMOTHERAPEUTIC STRATEGIES

II

D

ACUTE LYMPHOBLASTIC LEUKEMIA IN ADULTS

9 Treatment of Adult Acute Lymphoblastic Leukemia

Perspective 1

SPERO R. CATALAND AND RICHARD A. LARSON

CONTENTS

1. INTRODUCTION

Acute lymphoblastic leukemic (ALL) has served as the model for the cure of neoplasia by chemotherapy for several decades, but even now fewer than half of adult patients achieve long disease-free survival (DFS). Steady improvements in the cure rate for adults have been achieved through more accurate diagnoses, the use of intensive multiagent chemotherapy, careful attention to potential sanctuary sites such as the central nervous system (CNS), and the appropriate use of hematopoietic stem cell transplantation (SCT). Improved immunophenotypic, cytogenetic, and molecular techniques have identified subgroups of ALL that have differing outcomes with conventional therapies. Risk-adapted strategies have been designed to treat patients with high-risk characteristics aggressively while minimizing potential toxicity to patients with a favorable prognosis with standard therapies. Further progress will require that large numbers of uniformly evaluated patients be entered into randomized clinical trials, testing various total therapy components that heretofore have been added empirically. The ability to detect minimal residual disease during remission may identify patients who require alternative treatment and at the same time spare some patients unnecessarily prolonged treatments. New drugs will probably be required for major advances in the cure rate.

Treatment regimens for ALL have evolved empirically into complex schemes that use numerous agents in various doses, combinations, and schedules. Few of the individual components have been tested rigorously in randomized trials. Thus, it is difficult to analyze critically the absolute contribution of each drug or dose schedule to the ultimate outcome. Numerous nonrandomized trials have attempted to answer these questions, but multiple alterations in study design between sequential trials have made it difficult to assess the exact merit of each modification.

Despite obvious improvements in the outcome for adults with ALL, many important questions remain to be answered. Historically, most randomized clinical trials in ALL have been performed in the pediatric population, the results of which were extrapolated to adults. As our knowledge about the biology of ALL increases, it is becoming apparent that this disease differs markedly in children compared with adults. It is important to note that the efficacy of a treatment regimen in children does not necessarily translate into efficacy in adults.

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Table 1
Immunophenotypic Subtypes of ALL in Adults and Children

	Frequency (%)		*Disease-free survival (%) at 5 yr*	
	Children	*Adults*	*Children*	*Adults*
B-lineage ALL				
Burkitt-type	2	3–5	75–85	60–70
Precursor B	80–85	75–80	80	30–40
T-cell ALL	15	20–25	65–75	60

Table 2
Survival in CALGB Clinical Trials of Adult ALL by Cytogenetic Subset

		Overall survival		
Karyotype	*No.*	*Median (yr)*	*5-yr (%)*	*p value*[a]
Normal	79	2.9	37	
t(9;22)	67	1.3	11	<0.001
+8	23	1.3	12	0.004
t(4;11)	17	0.8	18	<0.001
–7	14	1.3	14	0.01
+21	32	1.5	26	0.06
del(9p) or t(9p)	28	1.3	38	0.58
del(12p) or t(12p)	11	6.8	82	0.10
t(14q11)	9	7.4	78	0.04

Abbreviations: CALCB, Cancer and Leukemia Group B.
[a]*p* value from the log-rank test for the difference in survival for each cytogenetic subset compared with patients with a normal karyotype.
Adapted from ref. *(2)*, with permission.

It is therefore imperative that adults be treated in prospective trials that address specific unanswered questions. In this chapter, we discuss several outstanding and challenging questions in the treatment of adults with ALL: (1) How does the heterogeneity of ALL impact on treatment outcome? (2) Can molecular monitoring for minimal residual disease be used to make clinical decisions? (3) Is there an optimal regimen for CNS prophylaxis? (4) Is postremission maintenance chemotherapy important for adults? (5) What is the optimal care plan for older adults with ALL? (6) How should isolated extramedullary relapses be managed? and (7) Which new agents hold promise for ALL in the future? Only through enrollment of patients in large, multicenter trials will these and other important questions be answered in the most convincing and timely manner.

2. HOW DOES THE BIOLOGIC HETEROGENEITY OF ADULT ALL IMPACT ON TREATMENT OUTCOME?

Historically, the treatment regimens used for adults with ALL largely evolved from those used effectively to treat childhood ALL. Nevertheless, the survival and cure rates seen in adults have not achieved the same high levels as those in children treated with similar regimens *(1)*. Although this may be owing in part to the inability of older adults to tolerate the side effects of intensive therapy, this factor alone does not completely explain the difference in outcomes. Clearly, ALL is a syndrome of biologically quite different subsets of lymphoblastic disorders that occur with different frequencies in children and adults (Table 1). Even within the adult population, the spectrum of ALL changes with increasing age.

Adults are more likely than children to present with acquired genetic features that are associated with adverse outcomes. This is perhaps best exemplified by Philadelphia chromosome positive (Ph+) ALL, a disease that is probably not curable in adults when treated with conventional chemotherapy alone. Among adults, as many as one-third of patients overall will have t(9;22), compared with < 5% of children, and the incidence may be even greater in patients older than 50 yr. A recent prospective cytogenetic study performed by the Cancer and Leukemia Group B (CALGB) showed that t(9;22), t(4;11), -7, and +8 were each associated with a poor outcome in adults (Table 2) *(2)*. One or more of these unfavorable cytogenetic features were present in 39% of the 256 patients. In addition, adults are less likely to present with the favorable cytogenetic features of hyperdiploidy (< 2% of adults) or t(12;21) (*TEL/AML1* rearrangement), subsets known to have a good prognosis in children.

There also appear to be considerable differences between adults and children in how specific chemotherapeutic drugs are metabolized. For example, methotrexate (MTX) has been an important drug in the treatment of childhood ALL. Some data indicate that pediatric B-lymphoblasts accumulate higher intracellular concentrations of MTX and long-chain MTX polyglutamates, both in vitro and in vivo, than do adult lymphoblasts, and this may account in part for the poorer responses seen in adults compared with children *(3)*. This observation provides evidence that lymphoblasts in adults are biologically different from childhood lymphoblasts, a fact that is important to remember in the design of clinical trials. It should not be assumed that efficacy in childhood ALL will translate similarly in adults.

With current immunophenotyping and cytogenetic or molecular diagnostic techniques, adults with ALL can be divided into favorable-, intermediate-, and poor-risk categories. In this way, appropriate risk-adapted therapies can be prospectively evaluated in patients at high risk of treatment failure, whereas patients with a more favorable prognosis are spared the toxicity of overly aggressive therapies.

In a recent analysis, the following good-risk features were defined: age younger than 30 yr, white blood cell (WBC) count at presentation < 30,000/μL, achievement of remission induction in < 4–6 wk, and no adverse cytogenetics [i.e., t(9;22), t(4;11) or other 11q23 abnormality] (Table 3) *(4,5)*. When > 400 adults enrolled on CALGB studies 8811 and 9111 were analyzed, > 90% of patients younger than 30 yr of age had a complete remission (CR) *(6,7)*. Their 3-yr overall survival was estimated to be 64%. Patients with T-lineage disease (T-ALL) also had a good outcome in these studies, with a 3-yr overall survival rate of approximately 65%. However, even this group of favorable-prognosis T-ALL patients can be further subdivided on the basis of surface marker expression of specific T-cell antigens.

Czuczman et al. *(8)* separated patients with T-lineage ALL into those that expressed one to three, four or five, and more

than six T-cell antigens. T-ALL patients expressing only one to three antigens had estimated 3-yr DFS and overall survival rates of 17 and 30%, respectively, compared with 86% for both 3- yr DFS ($p = 0.003$) and overall survival ($p = 0.004$) for patients who expressed six or more T-cell markers. The same early or pre-T-cell subset with expression of three or fewer antigens can also be identified by the presence of cytoplasmic CD3 (cCD3). From these observations, we anticipate that the group of T-ALL patients expressing multiple mature T-cell antigens will do well with current intensive induction and consolidation regimens. Therefore, further investigations of this subgroup of favorable-risk patients should focus on novel postinduction strategies that could potentially minimize toxicity while maintaining the same or better rates of DFS. For example, the hypotheses that such favorable T-ALL patients require 2 yr of prolonged maintenance chemotherapy or that they require cranial radiotherapy (CRT) for CNS prophylaxis should be tested in randomized trials.

In the same analysis by the CALGB, poor-risk patients were defined as those with adverse cytogenetics, B-lineage disease, and WBC count >100,000/μL at presentation, or age older than 60 yr *(4,5)*. Patients with t(9;22) or molecular evidence of the *BCR/ABL* fusion gene were estimated to have a <20% survival rate at 3 yr, and there do not appear to be any long-term survivors following chemotherapy alone. Almost all Ph+ ALL cases have a precursor B-cell phenotype, and most coexpress CD19, CD10, and CD34 *(8)*. Patients with WBC counts >100,000/μL at diagnosis had a median survival of only 11 mo compared with >44 mo for patients with a WBC count <30,000/μL *(6)*. Interestingly, hyperleukocytosis did not affect the favorable prognosis of T-ALL patients in the CALGB series, although it was an adverse feature in the larger German series of T-ALL cases *(9)*. Thus, using standard approaches, the group of patients with precursor B-cell ALL and high WBC counts has a very poor outcome, and they should be candidates for novel therapies. Patients able to tolerate dose-intensive therapies should be referred for allogeneic transplantation in first CR if a suitable donor is available *(10)*. Patients without a histocompatible donor would be appropriate candidates for investigational therapies after successful remission induction.

In summary, it has become increasingly clear that ALL is a heterogeneous disease with biologically distinct subsets that should be approached individually. As mentioned earlier, T-cell ALL already has a 3-yr survival rate >60% when treated with current intensive induction and consolidation regimens *(5–7)*. In addition, there is evidence that this subset of patients may gain additional benefit from greater exposure to cyclophosphamide and cytarabine *(11,12)*. Similarly, patients with Burkitt cell ALL (mature B-ALL) clearly benefit from dose-intensive treatment with alkylating agents and high-dose MTX and can be cured with only 21 wk of total therapy *(13,14)*. These patients must be accurately identified to have Burkitt's leukemia at diagnosis and treated independently of other ALL subtypes. Clinical trials must now account for different subsets of patients by evaluating different arms of postinduction therapy, providing risk-adapted therapy to patients known to be at high risk of treatment failure, and protecting patients with a good prognosis from overly aggressive or prolonged therapy.

Table 3
Favorable Features in Adult ALL

	CR (%)	*3 yr DFS (%)*	*3 yr overall survival (%)*
Age < 30 yr	94	51	69
WBC < 30,000/μL	88	51	59
T-cell ALL	97	63	69
CR within 30 d	—	51	59

Abbreviations: CR, complete remission; DFS, disease-free survival; WBC, white blood cell.
Adapted from ref. *(6)*, with permission.

Long-term follow-up beyond 3–5 yr is required to assess the efficacy of these novel strategies.

3. CAN MOLECULAR MONITORING FOR MINIMAL RESIDUAL DISEASE BE USED TO MAKE CLINICAL DECISIONS?

With the advent of sensitive molecular diagnostic techniques, decisions regarding optimal care for individual patients have become increasingly complex. The use of the reverse transcriptase-polymerase chain reaction (RT-PCR) assay allows for detection of levels of residual leukemia far below those detected by morphologic or cytogenetic techniques. Ideally, the persistence of molecular markers could be used to identify those patients destined to relapse long before overt clinical leukemia recurs. This would allow for earlier treatment at a time when the patient still has a minimal disease burden. For example, molecular markers could be used to evaluate patients in clinical remission to identify those patients who should be recommended for allogeneic SCT based on their burden of occult disease. As yet, however, our experience with molecular detection of residual disease is not sufficient to make such a clinical decision.

Initial studies showed the feasibility of using RT-PCR to amplify the mRNA of fusion genes that result from chromosome translocations present at diagnosis and demonstrated that the persistence of these transcripts after therapy portends a high risk of relapse *(15)*. In a prospective CALGB study of 18 Ph+ ALL patients receiving standard chemotherapy, the achievement of a negative result in the RT-PCR assay for *BCR/ABL*, even if transient, was associated with a significantly better survival than that observed among patients who remained persistently RT-PCR positive *(16)*. Patients with Ph+ ALL have been followed after allogeneic SCT for the persistence of the *BCR/ABL* transcript. Radich et al. *(17)* found that the relative risk of relapse was 11.2 times higher in patients with detectable p190 *BCR/ABL* transcripts after transplantation compared with those without detectable transcripts. By contrast, the detection of p210 *BCR/ABL* transcripts after transplantation was not predictive of relapse.

At the same time, recent work has demonstrated that the mere presence of the original clonal molecular marker, either a chromosomal translocation or a rearranged immunoglobulin heavy-chain (*IGH*) or T-cell receptor (*TCR*) gene during or at the completion of therapy, does not uniformly predict relapse *(18–20)*. Roberts et al. *(18)* concluded from a prospective study

of 74 children with ALL that evidence of the malignant leukemia clone can persist for several years after the completion of therapy, implying the presence of viable leukemia cells. The final 25 patients enrolled on that trial were prospectively evaluated for the presence of residual disease by PCR amplification of rearranged *IGH* genes. Seven of the 24 patients relapsed, and 17 remained in CR (1 patient was lost to follow-up). Of the 17 who remained in CR, 15 still had evidence of residual disease by PCR analysis.

Alternatively, these cells could represent a premalignant precursor of the original clone, lacking the additional genetic alterations necessary for the full malignant phenotype or marking a return to an earlier evolutionary stage of the leukemia *(20)*. In a prospective study in France, 178 children with ALL were followed serially with quantitative PCR to determine the implications of residual disease after treatment *(19)*. The risks of relapse and death were found to be 5.7- and 10-fold higher, respectively, in patients with molecular residual disease at completion of induction therapy compared with those without detectable disease. At additional time points later in their treatment program, the risk of relapse continued to be higher in patients with detectable disease: 7.3-fold higher after consolidation and 9.2-fold higher when measured after the late intensification therapy. In addition, the quantification of residual cells above a threshold of more than 1 in 10^3 cells was highly predictive of relapse at all time points. These authors concluded that residual leukemia (as measured by PCR amplification) during therapy was highly predictive of relapse and thus indicates a poor prognosis for the PCR+ group overall, but it was not always predictive for a relapse in individual cases.

Currently, there are several limitations to using RT-PCR-based monitoring of minimal residual disease. First of all, clonal evolution of the initial leukemia population can occur with loss of the tumor-specific marker, leading to false-negative findings in follow-up studies. Also, lymphoid tumors arising from a stem cell at a stage prior to the onset of *IGH* gene rearrangement may generate malignant daughter cells with multiple rearrangements. Theoretically, patients could then relapse with different rearrangements that again lead to false-negative results. Another difficulty lies in how best to use this information to allow clinical interventions prior to overt relapse. The optimal frequency for surveillance time points is not clear. Furthermore, a recent CALGB study (8763) demonstrated that bone marrow samples provide a more accurate picture of low levels of disease than do peripheral blood specimens (C. Reynolds, submitted manuscript). This will probably limit the frequency of postremission samples available for evaluation in clinical practice. Finally, the necessary reagents for PCR detection are still being developed. The development of individual patient-specific probes for *IGH* and *TCR* rearrangements is currently laborious and expensive.

The optimal use of these molecular studies for clinical decision making in adults with ALL remains to be defined. Clearly, a rising quantity of residual leukemia, as detected by quantitative PCR, seems to predict the imminent relapse of a patient's leukemia. Whether early detection of progressive disease will afford the clinician sufficient time to initiate therapy prior to overt clinical relapse has not yet been tested, nor has it been proved that early treatment of subclinical relapsed ALL leads to a better long-term outcome. The most difficult problem to address is the meaning of low levels of residual disease detected by PCR. Does this represent low levels of a viable leukemia clone that is under some form of growth inhibitory control, or are there other explanations? Analogous data have been reported in patients with acute myeloid leukemia (AML) and a t(8;21) and in patients with CML within 1 yr after allogeneic transplantation. In both situations, persistent PCR products have not been shown to be predictive of relapse *(21–23)*. By contrast, patients with acute promyelocytic leukemia (APL) who have rising levels of *PML/RARα* transcripts from t(15;17) do appear to suffer rapid relapse *(24)*.

Prospective studies with serial monitoring of molecular markers in adults are necessary to increase our understanding of what happens to ALL when patients are in CR and the significance of minimal residual disease to clinical outcome. Studies should evaluate samples of both peripheral blood and bone marrow taken at regular intervals to determine their concordance at times of both high and low disease burden. It is hoped that it will be possible in future clinical studies to use molecular detection as a surrogate marker of disease burden and thus come to conclusions more rapidly about the effectiveness of therapy without waiting for patients to relapse. Eventually, randomized studies wherein physicians react to these molecular data will be required to prove the clinical utility of minimal residual disease monitoring.

4. IS THERE AN OPTIMAL REGIMEN FOR CNS PROPHYLAXIS?

The CNS can be a sanctuary site for occult lymphoblasts because of the pharmacologic effects of the blood-brain barrier. Thus, attention to CNS prophylaxis is an integral part of the treatment of ALL. Generally, it is easier to eradicate occult disease and prevent CNS relapse successfully than it is to treat overt CNS leukemia when it occurs.

A relapse of ALL can occur either as an isolated event in the CNS or in combination with a recurrence in the bone marrow (systemic relapse). In four consecutive studies by the German Multicenter ALL study group (GMALL), 47 of 1433 patients (combined data) who achieved a CR experienced an isolated CNS relapse (3%), whereas 34 (2%) had a combined CNS and bone marrow relapse *(25)*. Risk factors for either an isolated CNS relapse or combined relapse were WBC >30,000/μL at diagnosis (8% vs 4%, $p = 0.007$), T-cell vs B-lineage ALL (8% vs 4%, $p = 0.006$), and lactate dehydrogenase (LDH) >500 U/L (8% vs 1%, $p = 0.001$). There was no prognostic significance to involvement of the cerebrospinal fluid at diagnosis, age, time to achieve CR, or the presence of the Ph chromosome, *BCR/ABL* gene, or t(4;11). Isolated or combined CNS relapses occurred more rapidly (median, 238 d) than did bone marrow relapses (median, 375 d, $p = 0.004$). Survival at 8 yr was only 12% for patients with an isolated CNS relapse and 9% for those with a combined CNS/bone marrow relapse. Although 83% of patients with an isolated CNS relapse achieved a second CR, only 4 of these 24 survived, and all 4 had received an allogeneic transplant.

Kantarjian et al. *(26)* also identified several characteristics that were associated with a higher risk of CNS involvement.

Patients with mature B-cell ALL (Burkitt type), LDH >600 U/L, or >14% of cells in the S + G2/M compartment of the cell cycle were significantly more likely to have CNS involvement ($p < 0.01$). There was also a trend toward increased CNS involvement in patients presenting with high WBC counts, but among the 153 patients included in the M.D. Anderson series, this was not statistically significant. High LDH levels are often seen at diagnosis with Burkitt-type ALL, and the WBC count is often very high in patients with T-ALL. Thus, patients in the highest-risk groups can be easily recognized by clinical means. Cell cycle analysis is not widely available and probably adds little predictive value.

The benefit from CNS prophylaxis in adults was demonstrated by a study reported by the Southeastern Cancer Study Group in 1980. After attaining CR, patients were randomized to receive either intrathecal (IT) MTX and cranial radiation (24 Gy in 12 fractions) or no CNS prophylaxis. The rate of CNS relapse was reduced from 32% to 10% in the patients who received chemoradiotherapy prophylaxis ($p = 0.03$) *(27)*.

The goal of CNS prophylaxis is to prevent leukemic involvement of tissues surrounding the brain, spinal cord, and nerve roots at a minimal cost and acceptable levels of toxicity. Therapy has been largely adapted from childhood regimens developed in the 1970s. Cranial irradiation combined with IT MTX has been the standard CNS prophylaxis for adults with ALL. Using this approach in children, the rate of CNS relapse ranged from 6 to 14% *(28–30)*. Craniospinal radiotherapy may be even more effective, but it causes considerable myelosuppression that can be long-lasting, as well as considerable growth retardation in children *(28,31,32)*. The spinal cord is rarely irradiated in adults.

In a recent analysis by the GMALL study group, a statistically significant decrease in CNS relapses was noted when the intensity of intrathecal and systemic therapy was increased *(25)*. In a group of patients judged to be at high risk of CNS relapse, patients who received nine additional doses of triple IT therapy (MTX, cytarabine, and hydrocortisone) plus high-dose systemic therapy had significantly lower rates of CNS relapse overall than did those who received only four IT injections of MTX plus 24 Gy of cranial RT (2% vs 7%, $p = 0.001$). Recently, the question has been raised as to whether cranial irradiation could be safely omitted in adults if systemic therapy using high doses of the antimetabolites MTX and cytarabine together with IT therapy were substituted in its place. Investigators at the M.D. Anderson Cancer Center evaluated four different CNS prophylaxis strategies in sequential adult ALL trials: (1) no prophylaxis; (2) high-dose systemic chemotherapy with MTX and cytarabine alone; (3) high-dose systemic chemotherapy (cytarabine) plus IT cytarabine; and (4) high-dose systemic chemotherapy (MTX and cytarabine) plus IT MTX and cytarabine *(33)*. Among patients judged to be at high risk for CNS relapse, the rates of relapse were found to be 42, 26, 20, and 2%, respectively ($p < 0.001$). The CNS relapse rates among patients in the low-risk group were not significantly different. This study confirmed that CNS prophylaxis is required and demonstrated that high-dose systemic chemotherapy together with IT chemotherapy can effectively replace cranial irradiation.

From the available data, it is clear that CNS prophylaxis is a necessary component of the total treatment of adults with ALL. High-dose intravenous cytarabine and/or MTX, combined with IT chemotherapy, appears to be as effective as cranial RT with IT chemotherapy in preventing CNS relapses. Although they are generally well tolerated, the neurologic sequelae of brain irradiation and high-dose MTX/cytarabine are not fully known. As a greater fraction of adults survive after treatment of ALL, these late complications could become more important. One might propose that a randomized trial be conducted to compare these two strategies for CNS prophylaxis. However, given the present low incidence of CNS relapse, approx 5%, such a trial would require a very large number of patients to show a significant difference. In the meantime, disease characteristics should continue to be analyzed to determine whether combined chemoradiation therapy or chemotherapy alone is optimal for different subsets of disease.

5. IS POSTREMISSION MAINTENANCE CHEMOTHERAPY IMPORTANT FOR ADULTS?

Maintenance (or continuation) chemotherapy has been shown to be an important element for the cure of pediatric ALL, but there have been no randomized trials in adults to assess its utility. Standard outpatient maintenance therapy typically utilizes 6-mercaptopurine and MTX with monthly pulses of vincristine and prednisone for 1–3 yr. The optimal duration for maintenance treatment is unknown. Several hypotheses for its mechanism of action and benefit are that: (1) the continuous presence of low-dose antimetabolites kills drug-resistant or slowly dividing leukemia cells as they reenter the cell cycle; (2) maintenance therapy modifies the host immune response, enabling it to destroy residual leukemia cells; and (3) maintenance therapy suppresses the proliferation of residual leukemia cells until senescence or apoptosis occurs, i.e., until the normal regulation of lymphocyte survival is restored.

Although there have been several studies in which maintenance chemotherapy was omitted, none were designed to evaluate maintenance chemotherapy *per se*. In the CALGB study 8513, all treatment was completed after only 29 wk *(34)*. Although the initial CR rates were similar, the median remission duration in this study was significantly shorter (11 mo vs 18 mo) compared with the immediately previous CALGB study (8011), which had used 2.5 yr of total therapy. Nevertheless, the median survival was 19 mo with the shorter treatment regimen compared with 16 mo in the earlier trial. Dekker et al. *(35)* also indirectly addressed this issue in a Dutch multicenter trial in which patients received a fairly intensive induction and consolidation regimen but no maintenance therapy. In that study, 130 patients with a median age of 35 yr (range, 16–60 yr) received four drugs for induction therapy (vincristine, prednisone, daunorubicin, and L-asparaginase) followed by three courses of consolidation therapy, with amsacrine, mitoxantrone, and etoposide each given together with high-dose cytarabine (2000 mg/m^2) in sequence *(35)*. All treatment ended after approximately 4 mo. The DFS rate was only 24% at 5 yr, and overall survival was 22%.

Although these results appear inferior to those obtained with other regimens that have used maintenance chemotherapy, the

poor outcome could be attributed to several factors apart from the lack of maintenance therapy. For example, the overall CR rate was rather low in this study (73%), despite an induction death rate of 14%. This was disappointing since all patients were younger than 60 yr, and two-thirds presented with a WBC count of <35,000/μL.

The Eastern Cooperative Oncology Group (ECOG) also reported on an intensive postinduction regimen without prolonged maintenance therapy (EST 2483) *(36)*. In that study, 89 patients received one course of high-dose cytarabine as intensification therapy followed by eight cycles of cyclophosphamide, doxorubicin, vincristine, prednisone, methotrexate, and L-asparaginase (MACHO) as consolidation therapy. The investigators concluded that this regimen did not improve the CR rate (69%) or the survival (median, 10 mo) for adults. However, they also stated that the shorter treatment did not provide worse results than they had previously achieved with a lengthy maintenance therapy. Neither of these conclusions remains valid in the face of much better contemporary results *(6,7,11,37)*.

In a recent pilot study, Swiss investigators treated 63 patients (median age, 27 yr; range, 17–72 yr) with an intensive induction and consolidation regimen that lasted only 4 mo *(38)*. After the initial two courses of therapy, patients were allocated to allogeneic SCT, autologous SCT, or one course of high-dose cyclophosphamide. The three postremission treatment alternatives were assigned on the basis of age, availability of an HLA identical sibling, and the achievement of a CR after the first two cycles of therapy. Patients who had not achieved CR were automatically assigned to the high-dose cyclophosphamide arm. No additional therapy was given after completion of the third course of chemotherapy (i.e., either a transplant- or high-dose cyclophosphamide). Of 63 patients treated, the DFS rate was 40% overall at 3 yr and did not differ among the three arms. However, in a recent update of this study, after 140 patients were entered, the 5-yr EFS rate was estimated to be only 21% overall and only 11% for the patients who received only the cyclophosphamide consolidation *(39)*. These results clearly do not settle the question of the necessity for maintenance chemotherapy, but they provide support for a larger, randomized trial to address the need for long-term maintenance therapy in the treatment of adults with ALL.

A critical issue is the differential benefit of maintenance chemotherapy within the different subsets of adult ALL. Patients who have a high risk of treatment failure even after intensive regimens such as allogeneic SCT, such as those with Ph+ ALL, are unlikely to benefit from low doses of maintenance chemotherapy. Each subset of patients [precursor B-cell, mature B-cell (Burkitt-type), pre-T-cell, or mature T-cell] may differ in the need for maintenance chemotherapy to cure disease. For example, patients with Burkitt-cell ALL have a high cure rate following short (18 wk), intensive chemotherapy regimens and do not thereafter appear to need further maintenance chemotherapy *(13,14)*. Similarly, one might predict that patients with T-cell ALL may not receive any additional benefit from long-term maintenance chemotherapy, but this hypothesis remains to be proved in a randomized study. In the above-mentioned Dutch study by Dekker et al. *(35)*, in which maintenance therapy was omitted, patients with T-ALL appeared to have a better 5-yr DFS than did patients with B-lineage disease (30% vs 22%), although the difference was not statistically significant *(35)*. Thus, the more favorable outcome of patients with T-ALL compared with B-lineage disease is still observed even in the absence of maintenance therapy. Nevertheless, large study groups that have utilized 2–2.5 yr of prolonged therapy now routinely report DFS rates that are twice as good for both subsets *(1,6,7,40)*.

The importance of maintenance therapy is likely to be inversely proportional to the effectiveness of the induction therapy. For patients who received suboptimal induction or consolidation therapies, extended maintenance therapy may in fact suppress the regrowth of leukemia for a time, although not completely eradicating it. In this situation, maintenance therapy would appear to improve the DFS rate but probably would not result in an increase in overall survival. For this reason, any prospective study analyzing the effectiveness of extended maintenance therapy cannot be considered independently of the induction and consolidation therapy used. To come to any reliable conclusions, studies randomizing patients to maintenance or no maintenance therapy must use identical and potent induction/consolidation regimens. Such a prospective, randomized trial addressing the role of maintenance chemotherapy in the treatment of adults with ALL would have to be quite large. It must be sufficiently powerful to determine which subsets of patients benefit from maintenance therapy. In this way, the cost and potential toxicity of maintenance chemotherapy could be spared for these patients who will not derive any benefit.

At this time, we can conclude that maintenance chemotherapy is not necessary for Burkitt-type ALL and probably does not benefit patients with Ph+ ALL, most of whom relapse despite intensive consolidation and maintenance therapy. Patients with other types of precursor B-cell ALL should continue to receive 2–2.5 years of total therapy. We speculate that most patients with T-ALL are probably cured with intensive induction and consolidation regimens (such as those used by the CALGB and GMALL study groups) and that prolonged maintenance therapy may not contribute to the cure rate, although this suggestion remains to be tested.

6. WHAT IS THE OPTIMAL CARE PLAN FOR OLDER PERSONS WITH ALL?

The management of ALL in older adults (older than 60 yr) is a considerable challenge. Increasing age is an independent adverse prognostic factor for remission induction, remission duration, and survival, because of both the biologic characteristics of the disease and the clinical conditions of the patients (Table 4). Older patients are more likely to have Ph+ ALL (perhaps as high as 40–50% of patients older than 50 yr) and less likely to have the more favorable T-cell ALL, a combination of adverse factors that increases the risk of treatment failure *(2,8)*. At the same time, older patients often have coexisting medical problems, making it difficult for them to tolerate the most effective, intensive therapies currently used for younger patients. These general observations are difficult to confirm because older patients are considerably underrepresented in the literature. Registry data report that the median age for ALL is

Table 4
Treatment Outcome for Adult ALL Patients Younger or Older Than 60 Years

				No. of patients (%)		
Reference	*Age group (yr)*	*No. of patients*	*Induction*	*CR*	*Resistant disease*	*Induction deaths*
MDA, 1994 *(41)*	<60	216	VAD	178 (82)	26 (12)	7 (3)
	≥60	52	VAD	30 (58)	15 (29)	6 (12)
GIMEMA 0183 *(67)*	<50	285	V, P, D, A	235 (82)	NA	NA
	≥50	73	V, P, D, A	49 (67)	NA	NA
CALGB 9111 *(6)*	<60	150	V, P, D, A, C	131 (87)	8 (5)	11 (7)
	≥60	35	V, P, D, A, C	27 (77)	2 (6)	6 (17)
MDA, 2000 *(40)*	<60	160	Hyper-CVAD	150 (94)	7 (4)	5 (3)
	≥60	44	Hyper-CVAD	35 (79)	2 (5)	7 (16)

Abbreviations: NA, data not available for subset analysis; VAD, vincristine, Adriamycin, dexamethasone; V, P, D, A, vincristine, prednisone, daunorubicin, L-asparaginase; C, cyclophosphamide; Hyper-CVAD, hyperfractionated cyclophosphamide, VAD; CALGB, Cancer and Leukemia Group B; MDA, M. D. Anderson Cancer Center; GIMEMA, Gruppo Italiano Malattie Ematologiche Maligne dell' Adulto.

two decades older than the 30–40 yr commonly observed in clinical trials. Older patients are often excluded from clinical trials, possibly because of physician concerns about their ability to tolerate chemotherapy. Thus, the small numbers of older patients who have been enrolled on clinical trials are likely to have been highly selected.

Most studies have documented poor outcomes in patients older than 60 yr. Although older patients treated with intensive regimens have shown good rates of remission induction (65–85% in some studies), the survival of these patients (17% overall at 3 yr in recent CALGB trials) has remained poor *(6,7)*. When less intensive regimens (such as VAD with vincristine, doxorubicin, and dexamethasone) have been used to treat older patients, CR rates have been lower, and overall survival has again been poor, with a 3-yr survival rate of 10% compared with 40% in patients younger than 60 yr *(41)*. In the aforementioned M.D. Anderson Cancer Center study using VAD for remission induction therapy, patients older than 60 yr had a lower CR rate than did younger adults (58% vs 82%) and a higher rate of resistant disease (29% vs 12%). Recently, improvements in the CR rate for older adults have been achieved by increasing the intensity of induction therapy together with improved supportive care *(1,7)*. Kantarjian et al. *(40)* recently reported on their experience with the hyperfractionated cyclophosphamide with VAD (hyper-CVAD) regimen. Patients received a total of eight courses of chemotherapy (hyper-CVAD alternating with high-dose MTX and cytarabine). All patients, excluding those with mature B-cell and Ph+ ALL, then received maintenance therapy with 6-mercaptopurine, MTX, vincristine, and prednisone (POMP) for a total of 2 yr. Of the 44 in this study who were 60 yr or older, 79% achieved a CR, and 25% were estimated to be alive at 3 yr. Even though 18% of the older patients had an ECOG performance status of 3 or 4, induction-related mortality was only 16%. Overall, this study suggests that an improved outcome can result when aggressive therapy is coupled with appropriate supportive care.

A primary difficulty in treating older patients is the frequent presence of comorbid medical conditions, reflected in a higher death rate during induction. Several studies have reported induction-related deaths in the range of 12–38% of patients *(6,41–43)*. Recent improvements in supportive care have the potential to lower this mortality. In CALGB study 9111, patients were randomized to receive growth factor support with filgrastim [granulocyte colony-stimulating factor (G-CSF)] or placebo during an intensive induction and consolidation treatment program *(7)*. Forty-one patients 60 yr of age or older plus 157 younger patients were enrolled. In this study, G-CSF was shown to have a significant effect on shortening the time to recovery of peripheral blood neutrophils. Following induction chemotherapy, the median time to recover an absolute neutrophil count (ANC) of >1000/μL was 16 d, compared with 22 d for patients receiving the placebo ($p = 0.001$). This may account in part for the reduction in induction-related mortality (10% vs 25%) among patients 60 yr of age or older. The CR rate in older patients randomized to receive G-CSF was 81%, compared with 55% for those receiving placebo ($p = 0.10$). The median survival, however, was still only 12 mo for patients 60 yr of age or older and did not differ by treatment assignment. The probability of DFS at 3 yr was 19%. It was concluded that the benefit of G-CSF support was most pronounced in patients who would otherwise have the slowest hematologic recovery (e.g., older patients) and that it was an important factor in the improved CR rate seen in older patients on this study. As yet, no other growth factor trial has enrolled a sufficiently large number of older adults to confirm these results.

Despite the higher rates of adverse disease features and resistance to therapy at initial presentation, an argument can still be made for aggressive induction therapy for older patients with ALL. Regardless of whether the physician's intent is curative or palliative, aggressive induction therapy should be instituted because it is most likely to restore normal hematopoiesis. After completion of induction therapy, consideration should then be given to the overall course and prognosis of the patient. Elderly patients with more favorable disease characteristics who are still in good health after induction therapy should continue with the same postremission therapy with curative intent as given to younger patients. In patients who are not felt to be able to tolerate additional therapy, the rapid restoration of normal hematopoiesis by the induction therapy still has considerable palliative benefit.

It is not clear what the optimal therapy is for older patients since regimen-related toxicity is a more important consideration for this subgroup. Because of these unsettled issues, it is important that studies be designed specifically for older patients and that they be enrolled in clinical trials. Only through prospective studies will we improve the treatment outcome for older patients with ALL.

7. HOW SHOULD ISOLATED EXTRAMEDULLARY RELAPSES BE MANAGED?

Extramedullary relapses that occur during treatment or after the completion of primary therapy pose several challenges. Two common sites for extramedullary relapses in adults are the CNS and the testes. Clinically, if the bone marrow were involved at the time of extramedullary relapse, the patient has had a systemic relapse of his/her disease and should be treated appropriately. Systemic relapses are probably best managed with reinduction chemotherapy followed by allogeneic transplantation, if possible. The issue regarding the most appropriate treatment becomes more difficult when the patient presents with an isolated extramedullary relapse. Similar to other aspects of care for adults with ALL, most of the available data come from clinical studies on children, and the results have been extrapolated to adults.

Historically, pediatric patients faced a dismal prognosis after a CNS relapse, with <20% of children becoming long-term survivors *(44)*. Investigators at St. Jude Children's Research Hospital concluded that hematologic relapse, rather than recurrent CNS events, was the main obstacle to cure after an isolated CNS relapse *(45)*. Therefore, recent approaches have emphasized early and intensive systemic chemotherapy, principally with antimetabolites coupled with intrathecal chemotherapy, aimed at treating occult systemic disease as well as the known meningeal involvement. Cranial irradiation was delayed until after the chemotherapy was completed. Ribeiro et al. *(45)* treated children with 6 mo of intensive reinduction and consolidation therapy, followed by craniospinal irradiation. Maintenance therapy was administered for a total of 1 yr if the CNS relapse had occurred after completion of the primary therapy, or for 2.5 yr if the isolated CNS relapse occurred during therapy. This strategy yielded a 5-yr EFS rate of approximately 70%, even though that 15 of the 20 patients had relapsed while still receiving their initial therapy. In a larger study conducted by the Pediatric Oncology Group, 83 children with isolated CNS relapses received 6 mo of reinduction and consolidation therapy, followed by craniospinal irradiation and maintenance chemotherapy for a total of 2 yr from the time of relapse *(46)*. The EFS rate at 4 yr was 71% for all patients and 83% for those whose first remission had lasted for 18 mo or more. This outcome approaches the results observed overall for children with newly diagnosed ALL.

Another site of extramedullary relapse is the testis. Among children, about 40% of isolated extramedullary relapses occur in this gland; in adults the frequency of testicular relapse is considerably lower *(47)*. Ovarian relapses are uncommon at any age. Again, relapse of ALL in the testis is probably a harbinger of systemic relapse. When pediatric ALL patients with isolated testicular relapse have been treated in the past with only local radiation therapy, there has been a high incidence of systemic relapse *(48–50)*. Using sensitive molecular techniques, evidence of leukemia can be found in bone marrow samples of some patients thought to have an "isolated" extramedullary relapse *(51–53)*. Nonetheless, it remains uncertain whether the bone marrow is the initial site of relapse, with leukemia cells then seeding the testis, or vice versa. This question was addressed in a study of 886 boys receiving treatment for ALL. The times to presentation with isolated testicular relapse or nonisolated testicular relapse (medians, 36 and 27 mo, respectively) were compared with those for boys with an isolated bone marrow relapse (median, 22 mo) *(54)*. The authors argued that because isolated testicular relapses as a group occurred later compared with bone marrow relapses, it would be unlikely for the bone marrow to be the source of the leukemia cells that spread into the testes and more likely that the testes were the source of residual lymphoblasts that then reseeded the marrow.

MTX has been shown to penetrate the testicular interstitium *(55)*. Also, the frequency of testicular relapse has decreased as the dose intensity of MTX has increased in pediatric trials, supporting the hypothesis that the testis is a sanctuary site and the origin of lymphoblasts responsible for local and systemic relapse *(56,57)*. In a report by Jahnukainen et al. *(54)*, a significant difference in EFS at 2 yr was observed for patients who had an isolated testicular relapse (63%), compared with patients who had an isolated bone marrow relapse (32%), again suggesting the occult persistence of residual disease rather than the emergence of a subpopulation of drug-resistant lymphoblasts. The implication is that the lymphoblasts in the isolated extramedullary site of relapse may remain as sensitive to therapy as at the time of initial diagnosis.

From the available data, it is clear that systemic as well as local therapy should be used for the treatment of isolated extramedullary relapses. Should this include SCT as systemic therapy? There are few data available for adults. Borgmann et al. *(47)* compared the effectiveness of autologous and allogeneic transplantation with that of chemotherapy and local irradiation in the treatment of isolated extramedullary relapse in 165 children up to age 19 (median, 9 yr old). In this study, 134 children (66 with isolated CNS and 58 with isolated testicular relapse) received polychemotherapy, including intermediate- or high-dose MTX. Cranial radiotherapy (18–24 Gy) was given to nonpreirradiated patients; 15–18 Gy were given to the remaining testis if it was histologically normal. Boys with bilateral testicular relapse had both testes either removed or irradiated with 24 Gy. Maintenance chemotherapy was then administered for 1 yr.

Thirty-one patients (median age, 11 yr) underwent bone marrow transplantation at a median of 152 d (range, 23–392 d) post relapse. Most of these patients had experienced their extramedullary relapse either within 18 mo of first remission (45%) or within 6 mo of the end of primary therapy (26%). Fourteen children received an autologous transplant and 17 an allogeneic transplant from an HLA-identical sibling.

The EFS rate at 5 yr was 50% for the children receiving chemotherapy and 36% for those undergoing transplantation ($p < 0.05$). These data suggest that, for children, transplantation

with its well-known regimen-related toxicity and late immunologic effects may not be necessary in the treatment of extramedullary relapse because chances for cure are not superior to those with salvage chemotherapy. Unfortunately, similar data for adults are unavailable.

These studies in children support the need for systemic as well as local therapy for adults with isolated extramedullary relapse of ALL, but the optimal regimen and intensity of treatment is not known, nor is it known whether an isolated extramedullary relapse in an adult has the same poor outcome seen with bone marrow relapse of ALL, or whether these adults can be rescued with the same high rate of success as children. There is a strong rationale for using drugs (MTX and cytarabine) known to penetrate the CNS and other extramedullary sites when given intravenously in high doses, in addition to local radiation and/or intrathecal therapy. Our practice is to individualize therapy based on the adequacy of primary treatment, the site and timing of relapse, and the patient's candidacy for allogeneic transplantation. Future clinical studies need to address these questions to determine the most effective regimen with acceptable toxicity.

8. WHICH NEW AGENTS HOLD PROMISE FOR ALL IN THE FUTURE?

The treatment of adults with relapsed ALL poses a serious challenge to the treating physician. Even when a matched sibling donor is available for allogeneic transplantation, patients still must achieve a second CR in order to have an acceptable rate of success. In Kantarjian et al.'s *(58)* review of various chemotherapy regimens that have been used for relapsed ALL, overall rates of second CR ranged from 30 to 83%. The wide variation was due more to differing patient and disease characteristics, especially the intensity of prior treatments, than to the effectiveness of the specific agents or combinations used for reinduction therapy. Among ALL patients receiving salvage therapy at the M.D. Anderson Cancer Center, the overall CR rate was approx 30%, and median durations of response and survival were only 6 and 5 mo, respectively. Kantarjian and colleagues *(58)* emphasized the point that even among those patients achieving a second CR, fewer than one-fourth would potentially be eligible for allogeneic transplantation because of the lack of a donor, inadequate insurance coverage, or rapid relapse of their disease.

Clearly, new agents are needed to improve the outcome of patients with relapsed ALL. Such agents have generally been evaluated in patients at first or later relapse, although mechanisms of high-grade drug resistance often develop rapidly after multiagent primary therapy. In order not to discard potentially useful agents, drug development strategies must allow rapid promotion of promising new drugs into front-line therapy, where the disease is likely to be more susceptible.

One of the newest agents presently undergoing clinical investigation is 2-amino-9-β-D-arabinosyl-6-methoxy-9H-guanine, or GW506U78 (Glaxo-Welcome). Compound 506U78 is a prodrug of arabinosylguanine (ara-G) that exerts its effects through dGTP and inhibition of DNA synthesis, leading to cell death. In early clinical trials in relapsed patients, the rates of complete and partial remission (PR) were 44 and 32%, respectively, in T-cell ALL, whereas only modest responses (25% PR rate) were observed in patients with B-lineage ALL *(59)*. The dose-limiting toxicity has been neurologic; 30% of patients experience grade 1 or 2 somnolence, but more severe encephalopathy and myelitis have also been seen at higher doses. The CALGB and SWOG are currently evaluating compound 506U at 1.5 g/m^2/d on a d 1, 3, and 5 schedule for adults with relapsed or refractory T-cell ALL and T-cell lymphoblastic lymphoma (CALGB study 19801).

Recent efforts have been directed toward developing therapies targeted to the specific features of ALL cells. One example is Campath-1H (Berlex; ILEX; LeukoSite), a humanized IgG anti-CD52 antibody. The CD52 antigen is expressed on 95% of all normal human lymphocytes as well as on most B- and T-cell lymphomas *(60)*. Relapsed or refractory chronic lymphocytic leukemia (CLL) patients experienced a 42% overall response rate (4% CR, 38% PR) to Campath-1H . Higher response rates were observed in previously untreated CLL patients, with eight of nine responding to Campath-1H (3 CR, 5 PR) *(61)*. Marked lymphopenia was the primary toxicity and led to opportunistic infections, which were more common in heavily pretreated patients *(60,61)*. As yet, data are meager on the effectiveness of Campath-1H against ALL. Campath-1H has potential utility both as postremission therapy in ALL and as a means of purging leukapheresis products ex vivo for autologous SCT. Future clinical trials will explore the optimal use of this monoclonal antibody in the treatment of ALL.

Another novel biologic agent in clinical trials is the B43 (anti-CD19)-genistein immunoconjugate. By its conjugation to a murine anti-CD19 antibody, genistein (a naturally occurring protein tyrosine kinase inhibitor) is targeted to the CD19-LYN complex *(62–64)*. Antibody binding results in apoptotic cell death. Uckun et al. *(64)* performed a pilot study of the B43-genistein conjugate in seven children and eight adults with relapsed or refractory ALL. Overall, the therapy was well tolerated, with no life-threatening side effects. One durable CR and two PRs were observed in this heavily pretreated group. The investigators noted that none of the patients who responded to therapy had had circulating blast cells prior to treatment, whereas 9 of the 10 patients who did not respond had had circulating ALL cells. It was hypothesized that the latter group was less likely to respond because of dilution of the immunoconjugate by the high number of peripheral blasts. Another difficulty was the development of human anti-murine antibodies, which limits repeated use of the murine immunoconjugate.

Another targeted biologic agent under investigation is imatinib mesylate (STI-571; Novartis). This inhibitor of the *ABL* tyrosine kinase has considerable activity against leukemias that overexpress the *ABL* or the *BCR/ABL* fusion gene products. The early results of STI-571 in patients with chronic myeloid leukemia (CML) in the chronic phase showed a high rate of hematologic and cytogenetic responses *(65)*. A small number of patients with relapsed Ph+ ALL or CML in the lymphoid blast phase have received STI-571 in phase I and II studies. The hematologic response rates were high, but the remission durations have been short. STI-571 has increased activity against CML cells in vitro when used in combination with interferon, daunorubicin, or cytarabine *(66)*. Although experi-

ence is still limited with this agent, it seems likely from initial studies that STI-571 would have activity in Ph+ ALL. Clinical trials to test combinations of STI-571 with chemotherapy and SCT for Ph+ ALL are now being designed.

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10 Treatment of Adult Acute Lymphoblastic Leukemia

Perspective 2

DIETER HOELZER AND NICOLA GÖKBUGET

CONTENTS

1. INTRODUCTION

Over the past two decades major developments in the biologic characterization and treatment of acute lymphoblastic leukemia (ALL) have led to improved understanding of underlying pathogenetic mechanisms, prognostic factors, and finally increased survival in at least some subgroups of adult ALL. Overall complete remission (CR) rates in adult ALL now range from 80 to 90%, with leukemia-free survival (LFS) rates of 30–40% in larger trials. For specific subgroups, such as B- and T-lineage ALL (B-ALL and T-ALL), LFS rates of >50% can be achieved, whereas for other entities, such as Philadelphia chromosome (Ph)/*BCR-ABL* positive ALL, the LFS rate is still <10%. The characterization of molecular aberrations and underlying pathogenetic mechanisms has provided both the foundation for strategies of detection and follow-up of individual disease markers—minimal residual disease (MRD) for example—and the option for individualized treatment approaches. Most recently these advances have culminated in the first clinical trials of "causal" molecular therapies.

ALL is not a uniform disease but consists of subgroups with characteristic biologic, clinical, and prognostic features. It is a rather rare disease in adults, which means that relevant questions can only be answered in prospective multicenter trials or at very large single institutions. Both facts have probably contributed to the situation in which major issues with regard to chemotherapy, stem cell transplantation (SCT), and prognostic factors remain unsolved (Table 1), as is discussed in this chapter.

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2. INDUCTION THERAPY

In contemporary trials, overall CR rates of 80–90% are achieved *(1)*. The available reports show a rather large variability, with generally lower CR rates in large multicenter trials, and in studies with higher median ages and earlier remission evaluation, compared with monocenter trials and studies with an upper age limit of 50–60 yr and CR rates that include the results of salvage therapies. Steroids, vincristine, and anthracyclines (doxorubicin or daunorubicin) are the backbone of induction therapy. There is also sufficient evidence that additional drugs such as cyclophosphamide, asparaginase, or cytarabine lead to higher CR rates than three-drug standard induction alone *(1)*. Optimization of induction chemotherapy not only aims to increase the remission rate but also seeks to improve remission quality. High-dose cytarabine (HDAC) has been used not only for remission induction but also to improve the prevention of central nervous system (CNS) relapse. A clear benefit of this strategy has not been demonstrated so far.

Table 1
State of the Art and Controversial Issues in the Treatment of Adult ALL

Topic	*State of the art*	*Controversial issues*
Induction therapy	Combination therapy including steroids, vincristine, vincristine, anthracyclines, and additional drugs	Antracycline intensity Role of L-asparaginase Role of high-dose cytarabine Indications for G-CSF
Consolidation therapy	Intensive, rotational consolidation therapy	Optimal combinations Subgroup adjusted treatment New drugs and monoclonal antibodies
Maintenance therapy	Maintenance therapy with 6-mercaptopurine and methotrexate required	Duration, intensity, drug combinations Role of maintenance in subgroups of ALL
Stem cell transplantation	Recommended in high-risk ALL	Indications in subgroups of ALL Indications for different BMT modalities (matched related, matched unrelated, autologous) Value of purging Graft-versus-leukemia effects in ALL New modalities
Prognostic factors	Age, WBC count, late achievement of CR, t(9;22)/*BCR-ABL* and t(4;11)/*ALL1-AF4* are adverse prognostic factors	Prognostic factors for subgroups of ALL New prognostic factors (immunophenotype, cytogenetics, and molecular markers) Minimal residual disease

Abbreviations: CR, complete remission; G-CSF, granulocyte colony-stimulating factor; WBC, white blood cell; BMT, bone marrow transplantation.

Up-front application of HDAC before conventional induction led to remission rates of 75 and 85% in two studies *(2,3)*, but long-term results are apparently not superior to those of other studies. Secondary application of HDAC after conventional induction was associated with intermediate remission rates of 67–77% (summarized in ref. *1*), with the exception of the hyper-fractionated cyclophosphamide with vincristine, Adriamyein, and dexamethasone (hyper-CVAD) regimen, which yielded a remission rate of 91% (4).

Therefore, several other new approaches are currently under investigation. One important issue is the type, schedule, and intensity of anthracycline therapy. Whereas formerly many regimens were based on weekly applications *(5)*, most trials now include a 2- or 3-d schedule that may be repeated after 1–2 wk. Daunorubicin is used in most studies, with a dose ranging from 30 to 60 mg/m^2 (Table 2). A higher dose intensity of anthracyclines may be associated with higher remission rates, as indicated by a literature review *(6)* and by remission rates, >90% in a small series *(7)*, although this result could not be reproduced in a large multicenter study yielding a remission rate of only 76% with the same regimen *(8)*. High dose intensity of anthracyclines may also be associated with increased induction mortality *(9)*. Thus, it remains important to show that intensive anthracycline treatment is feasible in multi-institutional studies and that its antileukemic effects are not outweighted by a higher mortality rate.

A more rapid reduction of tumor burden may also be achieved by early use of cyclophosphamide (Cp). Thus, high remission rates were achieved with fractionated Cp as part of the hyper-CVAD regimen *(4)*, and a reduction of treatment failure—particularly in T-ALL—was achieved with Cp pre-

Table 2
Intensification of Anthracyclines in Recently Published Studies in Adult ALL

		Anthracycline			
Author	*No.of patients*	*Preparation*	*Dose (mg/m^2)*	*Duration*	*Interval*
Dekker et al., 1997 *(10)*	130	DNR	40	1 × 3 d	—
Linker et al., 1997 *(11)*	62	DNR	60	1 × 3 d	—
Larson et al., 1998 *(12)*	198	DNR	45	1 × 3 d	—
Todeschini et al., 1998 *(7)*	60	DNR	30	2 × 3 d	11 d
Hallbook et al., 1999 *(3)*	120	DNR	30	1 × 3 d	—
Takeuchi et al., 1999 *(13)*	285	DOX	30	2 × 4 d	4 d
Bassan et al., 1999 *(9)*	80	IDA	10	1 × 2 d	—

Abbreviations: DNR, daunorubicin; DOX, doxorubicin; IDA, idarubicin.

treatment in an Italian study *(14)*. Several study groups, including the German Multicenter Study Group for Adult ALL (GMALL), are currently investigating the role of dexamethasone (Dx) instead of prednisolone in induction therapy. Dx attains a longer half-life in the cerebrospinal fluid (CSF) and possesses greater cytotoxicity. In a randomized study in childhood ALL comparing Dx and prednisolone during induction

therapy, the rate of CNS relapses was significantly lower with Dx (14% vs 26%) *(15)*. The known side effect of Dx, bone marrow necrosis, might be overcome by shorter applications of the drug than were used in earlier trials.

Although it has been demonstrated in only one randomized trial *(16)*, asparaginase (Asp) may have an important role in induction therapy. It may not contribute to a higher remission rate but rather to a longer remission duration. In adults, Asp is associated with various side effects such as liver toxicity and coagulation disturbances, which may lead to treatment interruptions during induction and hence a lower dose intensity. Thus, the timing of Asp application and schedule of administration remain to be established. PEG-L-Asp, a new preparation of *Escherichia coli* Asp synthesized by conjugation to polyethylene-glycol, has a significantly longer half-life, 5.7 d, than either native *E. coli* Asp (1.2 d) or *Erwinia* Asp (0.65 d) *(17)*. One application of PEG-Asp may replace 2 wk of treatment with conventional Asp, and PEG-Asp is apparently not associated with higher toxicity *(18)*, compared with native *E. coli* Asp. The longer half-life may be exploited to design shorter induction regimens with higher dose intensity. Whether this application would translate to a higher rate and quality of remission remains open to question.

Intensification of induction therapy is clearly associated with prolonged and more severe neutropenias. Therefore the early and prolonged application of granulocyte colony-stimulating factor (G-CSF) together with cytotoxic chemotherapy, may be important for achieving a higher dose intensity and reducing infection-related morbidity and mortality. Several studies have demonstrated that G-CSF can be safely administered in parallel with induction therapy in ALL and that the duration of neutropenia can be significantly reduced *(12,19)*. There was also a higher remission rate (90% vs 81%) and a lower induction mortality (4% vs 11%) in patients treated with G-CSF compared with placebo *(12)*. Whether this addition will contribute to an improvement in the LFS rate remains an open question. Studies of minimal residual disease (MRD) have convincingly shown that molecular remissions after induction therapy are associated with a very favorable prospect for long-term survival. Thus, dose intensification is required to achieve a higher rate of molecular remissions. In adult ALL, however, the major challenge is to avoid a parallel increase in treatment-related mortality. The risk of early mortality increases with age, and differential approaches are probably needed in older patients (see below).

3. POSTREMISSION THERAPY

In postremission therapy of adult ALL, SCT plays an increasingly important role. The contribution of consolidation therapy before SCT and the intensity, elements, and duration of consolidation therapy in patients who are not candidates for SCT remain to be defined.

3.1. Consolidation Chemotherapy

A variety of cytostatic drugs are administered in different combinations during consolidation therapy of adult ALL. The major aim is to use non-cross-resistant drugs to circumvent the expansion of resistant subclones. It is, however, impossible to assess the beneficial effect of single elements of multidrug combination regimens. Only overall effects of a defined treatment approach (including also induction therapy) may be evaluated. Retrospective analyses of published studies in adult ALL generally show a superior outcome in trials implementing intensive rotational multidrug consolidation therapy compared with those without such therapy. The results of randomized studies of consolidation therapy are inconclusive (Table 3), possibly in part because of low patient numbers, less intensive induction therapies, and the lack of subgroup analyses. In one large randomized trial, there was a reduction of relapse risk in patients receiving early and late intensification (37% LFS) vs those without these phases (28%) *(20)*. Two other randomized trials comparing conventional and intensified consolidation did not show an advantage for intensified treatment *(21,22)*. Whether this observation will be confirmed with longer follow-up remains to be seen. Particulary for B-precursor ALL, a longer observation period may be necessary to assess the impact of intensive consolidation therapy on late relapses.

3.2. High-Dose Chemotherapy

Either HDAC or methotrexate (HDMTX) has been used to overcome drug resistance and to achieve therapeutic drug levels in the CSF. The general impression is that the inclusion of HDAC, or HDMTX, or both might be beneficial *(1)*. The most favorable results (45–55% LFS) were again reported from smaller studies with prolonged intensive consolidation and maintenance therapy *(7,11,23)*. One study showed a particular benefit for B-lineage ALL with an LFS rate of 47% at 5 yr *(11)*. It remains to be proved whether this type of intensive and prolonged chemotherapy will be accepted by physicians and adult ALL patients and whether it would be feasible in larger national studies.

3.3. Maintenance Therapy

Maintenance therapy consisting of 6-mercaptopurine (MP) and MTX, augmented with vincristine and prednisone or more intensive cycles, is usually administered for a total treatment duration of 2–3 yr. It can be clearly stated that maintenance therapy cannot be replaced with a more intensive induction/consolidation therapy, at least not in patients who did not receive early SCT *(10,24,25)*. On the other hand, no large trial has demonstrated a clear advantage for patients treated with intensive maintenance therapy *(26)*. Furthermore, subgroup-specific differences have to be considered. Whereas, in B-ALL, maintenance therapy is apparently not required, the optimal duration and intensity of maintenance therapy in T-ALL remain open to question. In B-precursor ALL, omitting maintenance therapy is not recommended at present because of the very prolonged relapse risk. It is hoped that continued evaluation of MRD will provide a rational basis for decisions on the treatment of subgroups, particularly the intensity and duration of maintenance therapy (see below).

4. NEW APPROACHES FOR CONSOLIDATION THERAPY

4.1. New Cytostatic Drugs

Only a few new cytostatic drugs show promising effects in ALL (see overview in ref. *1*). Purine analogs such as fludarabine and cladribine, which were primarily used in low-grade B-cell

Table 3
Randomized Studies of Consolidation/Maintenance Therpay in Adult ALL

Author	*Year*	*Question*	*No. of patients*	*CR rate (%)*	*LFD rate (%)*	*Result*
Ellison et al *(27)*	1991	Postinduction intensification vs none: AC, D vs M, MP	277	64	29	No difference
Cuttner et al. *(24)*	1991	Mi vs D in consolidation	164	64	18	No difference
Stryckmans et al. *(28)*	1992	Conventional vs intensified consolidation: AC, C vs AC,C,A,M,TG	106	74	35	No difference
Fiere et al. *(29)*	1993	Allo-SCT for all patients with donor Auto-SCT vs chemotherapy in remaining patients	572	76	24	Superior outcome for allo-SCT in high risk patients No difference between auto-SCT and chemotherapy
Attal et al *(30)*	1995	Allo-SCT for all patients with donor Auto-SCT in remaining patients	135	93	44	Superior outcome for allo-SCT and patients with donor
Mandelli et al. *(26)*	1996	Conventional vs intensified consolidation: MP,M,V,P vs MP, M, V, D, P, IdAC, VM	358	79	27	No difference
Mandelli et al. *(21)*	1996	Conventional vs intensified consolidation + maintenance: MP, M, V, P vs C, AC, V, D, P, Mi, VM, HdM, IdAC, Dx	767	82	34	No difference
Durrant et al. *(20)*	1997	Early and/or late intensification (V,D,VP,AC,TG,P): no intensification vs early vs late vs both	618	82	28	Worst outcome without intensification Best outcome with early + late intensification
Ribera et al. *(22)*	1998	Conventional vs intensified maintenance: MO,M vs VD,Mi,P,A,C,VM,AC	110	86	44	No difference
Rowe et al. *(31)*	1999	Allo-SCT for all patients with donor Auto-SCT vs chemotherapy in remaining patients	920	89	NR	Allo-SCT superior for all subgroups Auto-SCT vs chemotherpay not reported

Abbreviations: AC, cytarabine; C, cyclophosphamide; A, asparaginase; M, methotrexate; TG, thioguanine; MP, mercaptopurine; V, vincristine; P, prednisone; D, danorubicin; IdAC, intermediate-dose AC; VM, teniposide; Mi, mitoxantrone; HdM, high-dose methotrexate; Dx, dexamethasone; LFS, leukemia free survival; NR; not reported; CR, complete remission; allo-SCT, allogeneic stem cell transplantation; auto-SCT, autologous SCT.

malignancies, have been evaluated in relapsed ALL. Of these, the combination of fludarabine and HDAC (FLAG regimen) has shown promising results in relapsed Ph/*BCR-ABL*-positive ALL. Compound GW506U78 (9-β-D-arabinosylguanine), a prodrug of arabinosyl-guanine (araG), exerts specific cytotoxicity toward T-lymphoblasts and may offer an additional treatment option for relapsed/refractory T-ALL and T-lymphoblastic lymphoma.

4.2. Monoclonal Antibodies

Antibody therapy is an attractive alternative approach to the treatment of adult ALL, since options for intensification of chemotherapy are limited owing to toxicity. Moreover, the different mechanisms of action of such treatment can lead to therapeutic effects against resistant subclones. Antigens on ALL blast cells, such as CD20, CD19, CD22, CD33, CD3, CD7, and CD52, offer several targets for monoclonal antibodies (MAbs). Thus, CD20 is expressed in one-third of B-precursor ALL cases and most cases of mature B-ALL and Burkitt's lymphoma. This provides a rationale for treatment with anti-CD20, a generally available antibody that has produced promising effects in combination regimens for high-grade B-cell lymphomas.

The MAb Campath-1H (anti-CD52) showed clinical activity in patients with relapsed adult ALL, with reduction in leukocyte counts, clearance of peripheral blast cells, and one partial remission in five patients *(32)*. Because CD52 is expressed in most lymphoblastic cells and to a higher degree in T- compared with B-lymphoblasts, clinical evaluation in larger patient cohorts is needed. Other antibodies (e.g., to CD19, CD3, CD7) have also been evaluated in small series of patients with relapsed/refractory ALL. They have shown limited efficacy but probably could not be expected to secure significant remission rates in overt disease treated with antibody only. Therefore, application of MAbs in *de novo* ALL with MRD or in combination with chemotherapy may be more promising. Thus, anti-B4-blocked ricin (anti-B4-bR), a protein toxin conjugated to the anti-CD19 MAb, was used as part of consolidation therapy after induction and one block of conventional consolidation in adult CD19-positive ALL. So far, a clinical benefit of this approach as part of a multiagent regimen could not be demonstrated, and there was only little impact of anti-B4-bR on MRD levels *(33)*. Nevertheless, MAb treatment in ALL deserves further clinical investigation. The potential application could, as in non-Hodgkin's lymphoma (NHL), be used as single-agent therapy or in combination with chemotherapy during induction and/or consolidation. It would be of particular interest to evaluate antibody therapy as maintenance in patients with MRD or in patients who are not eligible for conventional chemotherapy (e.g., owing to age or clinical infections).

4.3. Risk-Adapted Consolidation Therapy

In the future, attempts to tailor consolidation and maintenance therapy to specific subtypes of ALL should be refined. This approach has already been successful in mature B-ALL and in T-ALL. Furthermore, MRD evaluation provides the unique option to evaluate the particular effects of consolidation cycles in individual patients. Approaches to risk-adapted consolidation are discussed later in this chapter.

5. STEM CELL TRANSPLANTATION

Allogeneic stem cell transplantation (allo-SCT) from sibling donors in patients with high-risk ALL is a generally accepted treatment approach. There are, however, different current definitions for high-risk features, and in some studies allo-SCT is restricted to patients with very-high-risk features, such as Ph/*BCR-ABL*-positive ALL. Two major general approaches to the allocation of patients to allo-SCT are under investigation. One is to refer all patients with a matched sibling donor to early allo-SCT. The other is to offer this procedure only to patients with high-risk features. The role of autologous SCT (auto-SCT) compared with chemotherapy and the potential benefits of matched unrelated SCT remain to be defined.

5.1. Allogeneic SCT from Sibling Donors

The survival rate among adult ALL patients undergoing allo-SCT from sibling donors in first CR is approximately 50%, with a very wide range of published results. The general impression is that results from specialized transplantation centers are superior to those achieved in multicenter studies or collected in registries. Nevertheless, the overall LFS rate after allo-SCT seems to be superior to that achieved with chemotherapy alone. However, for an appropriate comparison, it is necessary to adjust for age, other risk factors, general condition, and particularly time to transplantation, since patients who actually received SCT may represent a selected cohort of younger patients in good general condition, in contrast to patients who were excluded because of early relapse. Furthermore, long-term quality of life should be taken into consideration in these comparisons.

A realistic estimation of the beneficial effects of allo-SCT may only come from prospective studies with "natural" randomization to allo-SCT for all patients with a suitable donor and an intent-to-treat analysis. In the so far largest randomized trial, allo-SCT was scheduled for all patients younger than 40 yr of age with a sibling donor *(29)*. The remaining patients were randomized (control group) to receive either auto-SCT or chemotherapy. In a recent update, the survival after allo-SCT was significantly superior (46%) compared with that of the control group (31%), primarily because of the higher survival in high-risk patients undergoing allo-SCT (37% vs 15% among controls). No significant difference was observed among standard-risk patients (46% vs 42%) *(34)*. These findings would support the strategy of offering SCT only to patients with high-risk features, particularly since the overall results of SCT are not superior to the best results with chemotherapy in standard-risk patients. However, only a few results are available on the results of allo-SCT in standard-risk patients. The ongoing ECOG/MRC trial is similar to the above-mentioned French study, in that allo-SCT is offered to all patients with sibling donors compared with randomization between auto-SCT or chemotherapy in patients without a suitable donor (control group). The LFS rate after 3 yr was 58% for patients who actually received allo-SCT compared with 39% for controls. Allo-SCT yielded a superior LFS rate for high-risk (57% vs 32%) and for standard-risk (71% vs 54%) ALL patients younger than 60 yr *(31)*. An intent-to-treat analysis of this study is awaited, as it is still unclear whether this approach leads to an improvement of overall results.

5.2. Allogeneic SCT from Matched Unrelated Donors

SCT from matched unrelated donors (MUD) is increasingly employed in adult ALL, but published results are still scarce. In early studies MUD-SCT was generally associated with a lower relapse rate—probably because of a more pronounced graft-versus-leukemia (GvL) effect—which was, however, nearly outweighed by a high treatment-related mortality *(35)*. With increasing experience, improved supportive care, better selection of donors, and management of graft-versus-host-disease (GvHD), a reduction in treatment-related mortality can be expected. One retrospective analysis of adult poor-risk ALL patients showed a promising survival of patients transplanted in first CR (40%) *(36)*.

5.3. New Approaches for Allogeneic SCT

GvL effects in ALL are less pronounced than in AML or CML. They are, however, appreciable, as indicated by a lower relapse risk in patients with GvHD compared with those without this complication *(37)*. In single cases, remissions could also be achieved in ALL patients with relapse after allo-SCT with donor lymphocyte infusions or interruption of GvHD prophylaxis. Donor lymphocyte infusions in combination with chemotherapy may therefore be worth further investigation, not only in patients with relapse after allo-SCT but also in patients with persistent residual disease after allo-SCT. In an ideal setting donor lymphocyte infusions could be adapted to the course of MRD and donor chimerism.

GvL effects are also used in non-myeloablative stem cell transplantation (NMSCT). Only a little published experience is available for adult ALL patients. In a pilot study conducted by the GMALL group, the procedure was feasible, and sustained remissions were achieved in single Ph/*BCR-ABL*-positive patients with contraindications for conventional SCT, such as older age or fungal infections. The results were clearly superior if non-myeloablative was administered as the first SCT approach and not in patients with relapse after conventional SCT *(38)*. Therefore, this technique may be an option for the large cohort of older patients with high-risk features who are not eligible for conventional allo-SCT.

Since the relapse rate after sibling allo-SCT is still high, new conditioning regimens are under investigation. Intensification of total-body irradiation (TBI) or chemotherapy is probably not feasible, but a more targeted approach with radiolabeled antibodies may be useful. Antibodies conjugated to radioemitters such as ^{131}I, ^{99}Tc, and ^{90}Y are capable of delivering additional irradiation doses rather specifically to hematopoietic tissues such as bone marrow and spleen. Radioimmunoconjugates are generally administered before conventional TBI and chemotherapy conditioning in an allogeneic or autologous setting. In a phase I study with ^{131}I conjugated to the murine anti-CD45 antibody in 44 patients with advanced leukemia, a favorable distribution with significantly higher estimated radiation doses to hematopoietic tissues compared with normal organs was achieved in 84% of the patients. Three of nine patients with relapsed or refractory ALL survived disease-free at 19–66 mo after SCT *(39)*.

5.4. Autologous SCT

Only a third of potential transplant candidates will have an HLA-identical sibling donor, and even after inclusion of MUD-SCT, a considerable number of patients will not be eligible for allo-SCT because of a lack of donors or other factors such as older age. Auto-SCT, which offers the attractive option of earlier treatment cessation compared with prolonged consolidation and maintenance chemotherapy, would be a reasonable option for these patients. In one analysis of the European Group for Blood and Marrow Transplantation (EBMT) registry data, the LFS rate after transplantation of autologous peripheral blood stem cells was 41% compared with 35% when bone marrow was used as the stem cell source *(40)*. These overall results are comparable to those obtained with chemotherapy alone. However, randomized prospective studies generally failed to demonstrate an advantage for auto-SCT *(34)*, and subgroup analyses are generally not available. In one Italian trial there was an advantage for auto-SCT, with a 5-yr LFS rate of 36%, compared with 17% for patients treated with chemotherapy alone *(9)*. The outcome for chemotherapy patients was, however, probably not the best possible result.

The mortality associated with auto-SCT is low (<10%), but the high relapse rate (>50%) remains a major problem. Relapses are most probably related to contamination of the graft with leukemic blasts. Several approaches have been taken to decrease the relapse rate. Chemotherapy before transplantation is an important issue, since patients with a lower tumor burden before transplantation have a lower risk of relapse. Purging of bone marrow or peripheral blood is another means of reducing tumor load and thereby the relapse risk. Evaluation of MRD in the graft allows an estimation of the effectiveness of purging. Two recent trials demonstrated effective reduction of residual blast cells by purging (median 1 log reduction). MRD of <5% in the graft was associated with a higher LFS rate after auto-SCT (87% vs 0%) *(41)*. A retrospective analysis of 52 ALL patients receiving auto-SCT in first CR showed a significantly higher LFS for purged (52%) compared with unpurged (13%) bone marrow grafts *(42)*.

Maintenance therapy after auto-SCT would be of particular interest in patients with MRD after transplantation. Several options — MP, MTX, interleukin-2 (IL-2) or interferon-α have been evaluated. No advantage was demonstrated for IL-2 with an LFS of 37% for patients actually receiving IL-2 compared with those not treated with this agent (37%) *(43)*. Maintenance with MP/MTX yielded a favorable overall survival of 56% at 5 yr in one study *(44)*, but this result has not been confirmed by other groups.

Taken together, these data show that auto-SCT deserves further exploration, particularly since there are still options for intensification of conditioning regimens (e.g., double transplantation), and MRD evaluation provides a method for tailoring additional treatment in individual patients.

5.5. Indications for SCT in Adult ALL

Since the results of large prospective trials are still awaited, the controversy over whether sibling allo-SCT should be offered to all patients with a suitable donor, or only to patients with high-risk features, cannot be resolved at present. In an ongoing study, the GMALL group is testing a plan of SCT indications that extend to patients with high-risk features and include sibling allo-SCT, MUD-SCT, and auto-SCT (Table 4).

Table 4
GMALL Approach to SCT Indications in Adult ALL

Stage	*Indication*	*SCT modality (priority)*
First CR	All high-risk patients < 55 yr within 3–4 mo from diagnosis; standard-risk patients if high-risk MRD status after 12 mo of chemotherapy	1. Allogeneic sibling 2. Allogeneic MUD 3. Autologous
Second or subsequent CR	All patients	See above
PR or beginning of relapse	SCT without additional chemotherapy if blasts in bone marrow < 30%	See above
HR but ineligible for regular allo-SCT	High-risk patients >55 yr or with contraindications for regular allo-SCT	NMSCT

Abbreviations: GMALL, German multicenter Study Group for Adult ALL; CR, complete remission; PR, partial remission; HR, high-risk; NMSCT, nonmyeloablative stem cell transplantation; MUD, matched unrelated donor; MRD, minimal residual disease; allo-SCT, allogeneic SCT.

For patients scheduled for auto-SCT, an additional consolidation cycle is scheduled in order to reduce the tumor burden.

6. PROGNOSTIC FACTORS

Various prognostic factors have been defined that significantly influence the remission rate and relapse risk in adult ALL. They have to be considered for comprehensive evaluation of treatment results, definition of risk-adapted treatment regimens, and indications for SCT. Patient characteristics at the time of diagnosis are available in all ALL patients and form the backbone of risk stratification in adult ALL. Age, white blood cell (WBC) count, and immunophenotype *(45)* are generally accepted risk features in adult ALL.

6.1. Age

An older age is associated with a lower remission rate mainly because of an increased rate of induction mortality (>20%) and is also associated with an inferior overall survival. Older patients, however, also show a higher relapse risk owing to an increased incidence of poor prognostic features, including Ph/*BCR-ABL* ALL and early T-ALL. Thus, >70% of patients older than 55 yr have common ALL, and of those >50% exhibit Ph/*BCR-ABL* ALL *(46)*. Older ALL patients show a wide variability in terms of general condition and comorbidity, and the stress tolerance of physiologic systems (e.g., hematopoiesis and liver function) generally decreases with age. Thus, a lower tolerance to chemotherapy associated with a lower dose intensity and a higher rate of complications (e.g., infections and organ toxicity) must be expected. Optimal supportive care, including the use of G-CSF, is certainly required. It is, however, not possible to define a cut-point in terms of age for feasibility of intensive chemotherapy, and there is increasing criticism toward adherence to predefined age limits and to exclusion of patients from curative treatment approaches solely based on age. Therefore, comprehensive measures for the assessment of biologic age are required.

One major challenge is to define dose-reduced but still curative regimens for older patients, since the incidence of ALL increases with age, and poor results in older patients have a major impact on the overall outcome of ALL. Furthermore, for older patients with high-risk features, alternative strategies—not based on intensification of chemotherapy—are urgently required. These include NMSCT, antibody treatment, and molecular therapeutic approaches such as STI571.

6.2. White Blood Cell Count

A WBC count greater than 30,000 or 50,000/μL at the time of diagnosis is associated with a higher relapse risk, even if this is the only risk factor. The adverse prognostic impact of a high WBC count may, however, be different in B- and T-lineage ALL. T-ALL patients generally have a higher WBC count at diagnosis, and therefore the cut-point for adverse prognostic impact may be higher (>100,000/μL) *(47)*. Nonetheless, a recent multivariate analysis by the GMALL group revealed that WBC count is no longer a relevant prognostic factor in T-ALL.

6.3. Immunophenotype

Immunophenotype defines subgroups of ALL patients with distinct clinical characteristics and different biologic subtypes of disease. Uniform criteria should be applied for definition of subgroups in order to make published results comparable. Table 5 shows the immunologic classification of adult ALL as used in the GMALL studies. B-precursor ALL has an incidence of 76% among adult ALL cases and can be classified into pro-B, common, pre-B, and mature B subtypes (Table 5).

6.3.1. Pro-B-ALL

Pro-B-ALL ($CD10^-$) is characterized by a high WBC count, a high incidence (>50%) of coexpression of myeloid markers (CD15/CDw65), and t(4;11). This subtype occurs frequently in infants and once was associated with a poor prognosis in adult ALL. With intensive chemotherapy including HDAC and mainly allo-SCT, the LFS rate could be improved to 40–50% *(48)*. The value of HDAC in pro-B-ALL is also supported by the higher sensitivity of blast cells in this disease compared with other ALL subtypes, as demonstrated in in vitro drug resistance tests *(49)*.

6.3.2. Common and Pre-B-ALL

Common and pre-B-ALL show a high incidence of the Ph/*BCR-ABL* rearrangement (30–50% depending on age), a higher median age compared with that in T-ALL and a slower but still resistant course of disease, with relapses (mainly in bone marrow) occurring for 5 yr and more after diagnosis. These patients can be allocated to a standard-risk group with an LFS rate of 40–50% and a high-risk group with an LFS rate of <20% *(45)*. Significant improvement in outcome could not be achieved in common/pre-B-ALL in the past decade. The best results were obtained in small series with the use of cyclic consolidation therapy including HDAC and HDMTX. HDMTX also contrib-

Table 5
Immunologic Classification of Adult ALL

Leukemia subtype	*Most important surface markers*	*Frequency (%)*	*Frequent cytogenetic aberrations*
B-lineage	HLA-DR$^+$, TdT$^+$, CD19$^+$	76	
Early pre-B	CD10$^-$	11	t(4;11)
Common	CD10$^+$	51	t(9;22) 9p aberr. 12p aberr. Hyperdiploid
Pre-B	CD10$^\pm$, cyIgM$^+$	10	t(1;19) t(9;22) Hyperdiploid
Mature B	TdT$^\pm$, CD10$^\pm$, sIgM$^+$	4	t(8;14) t(8;22) t(2;8)
T-lineage	TdT$^+$, cyCD3$^+$, CD7$^+$	24	
Early T	CD2$^-$, sCD3$^-$, CD1a$^-$	6	t(11;14)
Thymic T	sCD3$^\pm$, CD1a$^+$	12	t(10;14)
Mature T	sCD3$^+$, CD1a$^-$	5	9p aberr.

Abbreviations: TdT, terminal deoxynucleotidyl transferase.

uted significantly to the improvement of outcome in childhood B-precursor ALL *(50)*. The major difference is, however, that adult patients with B-precursor ALL show a higher incidence of poor prognostic features including known factors such as Ph/*BCR-ABL*, a lower incidence of hyperdiploid ALL and *TEL-AML1*-positive ALL, which are considered to be favorable subgroups, and several emerging factors such as unfavorable drug pharmakokinetics for MTX, as well as a likely higher incidence of drug resistance. The prognostic value of rare cytogenetic or molecular aberrations remains unclear.

Thus, without considerable improvement in the treatment results for common/pre-B-ALL, it will be difficult to improve the overall outcome in adult ALL. Attempts to achieve this goal are being made with intensive rotational consolidation therapy, including HDMTX and SCT for high-risk patients. Higher doses of anthracyclines given in induction and reaching a certain cumulative amount may also be associated with improved results *(51)*. Furthermore, a prolonged maintenance treatment appears to be required in B-precursor ALL. In this subtype, the evaluation of MRD seems to be of particular interest, since relapses in standard-risk B-lineage ALL cannot be explained by known prognostic factors. In future trials, treatment decisions may be based on MRD results.

6.3.3. Mature B-ALL

Mature B-ALL shows features similar to those of Burkitt's lymphoma and is characterized by L3 morphology and expression of surface immunoglobulins. The course of disease is generally rapid, with an extensive tumor mass often associated with organ involvement (e.g., abdominal lymphoma, CNS involvement, and others). In the past decade, considerable improvement of outcome was achieved by adaptation of intensive regimens developed for childhood ALL mainly based on fractionated Cp and HDMTX. Short cycles are administered in a rapid sequence, leading to LFS rates >50% *(52,53)*. Similar regimens are succesfully administered in Burkitt's lymphoma and other types of B-cell high-grade lymphoma such as large cell anaplastic lymphoma and diffuse large cell lymphoma. It became evident, however, that high doses of MTX (>1.5 g/m^2) are less feasible in adults than in children and frequently lead to severe mucositis with subsequent treatment delays. Further improvement may be achieved by inclusion of HDAC *(53)*. Immunotherapy—particularly with anti-CD20 antibodies—may be a promising approach as well since in most patients mature B-ALL expresses CD20.

Only a few data are available on the prognostic factors in B-ALL. An older age, a large tumor mass as indicated by multiple organ involvement, a high WBC count, increased LDH levels, and clearly slow or inadequate responses to therapy may be associated with an inferior outcome.

6.3.4. T-Lineage ALL

T-lineage ALL can be classified into early T, thymic, and mature T subtypes (Table 5). T-ALL patients are generally younger and show a high WBC count at diagnosis and frequently lymphomatous features including mediastinal tumors in 60% of the patients. These cases generally show a rapid progression with only few relapses after 3 yr. Because of the higher risk of CNS relapse, intensive CNS prophylaxis is required. Cp and cytarabine (ara-C) are apparently important drugs in T-ALL *(54,55)*. From childhood ALL studies there comes some evidence that HDMTX *(56,57)* and Asp *(58)* may be important drugs for consolidation therapy in T-ALL.

T-ALL is nowadays generally considered a favorable prognostic subgroup. In a large series covering several studies of the CALGB, the LFS rate in T-ALL after 3 yr (62%) was clearly superior to that in B-lineage ALL (42%) *(59)*. However, within T-ALL, additional prognostic factors can be identified. According to the GMALL experience, a high WBC count (>100,000/μL) and late achievement of CR are associated with a poorer outcome. The most relevant factor, however, was the immunologic subtype. Despite the major improvement of overall outcome in T-ALL in the last decade, the LFS rate of early and mature T-ALL is still poor (<30%). Early T-ALL is generally CD2 negative and shows a higher median age, lesser lymphomatous features (such as lymphadenopathy and mediastinal tumor), and a high incidence of myeloid antigen coexpression. This subgroup as well as patients with mature T-ALL probably has a poorer outcome *(47)*. Similar findings were reported for childhood ALL *(60)*. Therefore, these subgroups will be an indication for allo-SCT in future GMALL studies. Thymic T-ALL is characterized by CD1a expression and was the most favorable subgroup in the GMALL studies, with an LFS rate of 50–60%.

Treatment of mediastinal tumors is a specific issue in T-ALL. Only a few study groups have described mediastinal irradiation as a component of treatment protocols. In earlier GMALL studies, all patients with a mediastinal tumor at diagnosis received prophylactic mediastinal irradiation, resulting in

a very low rate of local recurrences. With intensification of chemotherapy, however, this procedure was associated with prolonged cytopenias and infectious complications. Whether restriction of mediastinal irradiation to patients with residual mediastinal tumor after induction therapy will provide a similar low rate of local recurrence is under investigation.

6.4. Cytogenetic and Molecular Aberrations

Chromosomal abnormalities are detected in >60% of adult ALL patients. These aberrations are not only correlated with distinct subtypes of ALL (Table 5) but also represent independent prognostic factors. Three study groups published the results of cytogenetic analyses in a total of 1049 patients *(61–63)*. The Ph/*BCR-ABL* and t(4;11) abnormalities were unanimously considered high-risk features. –7, +8, and hypodiploid ALL were classified as poor risk by some groups *(61)*, but this association remains to be confirmed in prospective studies of larger patient cohorts. 12p aberrations, t(10;14), and a high hyperdiploid karyotype were reported to be favorable prognostic features. It remains open whether cytogenetic abnormalities are independent prognostic factors, since many of them are associated with certain immunologic subtypes.

The CALGB suggested a stratification into three prognostic subgroups: poor [including t(9;22), t(4;11), –7, and +8], normal diploid, and miscellaneous (all other structural aberrations). The LFS rates were 11, 38, and 52% respectively *(61)*. This type of risk stratification requires further refinement, particularly with regard to the intermediate and favorable subgroup.

Overall, cytogenetics may add reasonable information for better definition of disease biology and risk stratification. However, the prognostic relevance of many specific cytogenetic aberrations remains unclear, as indicated by the large variability of reported outcomes mainly dependent on the treatment regimen administered. The major problem is the low incidence of most aberrations. This is also the reason why the overall clinical impact of these abnormalities is limited.

6.5. Molecular Genetics

The molecular detection of *BCR-ABL* and *ALL1-AF4* fusion genes, related to the translocations t(9;22) and t(4;11), respectively, is part of the standard diagnosis in adult ALL. The prognostic value of other molecular aberrations is less clear and has so far been evaluated only in small patient cohorts. Candidate genes for aberrations with possible prognostic relevance are the *TEL-AML1* fusion gene associated with the cryptic translocation t(12;21), which is associated with favorable prognosis in childhood ALL. Homozygotous deletions of the *p16* tumor suppressor gene are detected in 20–40% of adult and childhood ALL cases. They occur more frequently in T-ALL than in B-lineage ALL *(64)*, but the prognostic relevance remains so far unclear. Mutations of the tumor suppressor gene *p53* have an incidence of <10% in ALL, although one study in adult ALL found frequencies of 21 and 42% at relapse *(65)*. In T-ALL the incidence may increase up to 24% at relapse. From paired samples collected at diagnosis and at relapse, it became evident that only one of the patients with *p53* mutations at relapse showed similar mutations at diagnosis. Thus, the mutations of *p53* may have a role in disease progression in T-ALL. Furthermore, the outcome of T-ALL patients with *p53* mutations was inferior in terms of CR rate (33% vs 69%) and survival (13 vs 20.5 mo) after relapse compared with patients without *p53* mutations *(66)*.

The evaluation of the prognostic impact of single molecular aberrations requires prospective evaluation in larger patient cohorts with predefined treatment protocols. Furthermore, analysis should be performed in correlation with other prognostic factors (e.g., immunophenotype). At present the major impact of molecular analysis probably lies in a better understanding of pathogenetic mechanisms and hopefully the identification of targets for molecular treatment approaches (see below). DNA chip technology may in the future add significant information by screening for molecular aberrations in subtypes of ALL *(67)*.

6.6. Response to Treatment

The kinetics of blast cell reduction was so far mainly measured by the time required to achieve a complete remission. Adults with ALL not remitting within 4 or 5 wk fare poorly. *(45)*. The worse LFS rate for patients with a slowly induced CR may be explained by the presence of a more resistant blast cell population. Since early blast clearance is an important predictor of survival, response to chemotherapy is now evaluated as early as 2 wk after the start of chemotherapy or, in children, after 7 d of prephase therapy with prednisone.

6.7. Minimal Residual Disease

Molecular methods for monitoring treatment response in individual patients have gained increased importance in recent studies. MRD refers to residual leukemic blast cells that cannot be detected by microscopic examination, of the bone marrow smear. With conventional microscopic examination, the detection limit for residual blast cells is 1–5%, and after achievement of hematologic CR, further evaluation of the individual course is not possible. With new methods, the detection of leukemia by specific phenotypes (LAIP) using flow cytometry (reviewed in ref. *68*), translocation breakpoints [e.g., *BCR-ABL*, *E2A-PBX1*, *MLL-AF4*, and *TEL-AML1*) with fluorescence in situ hybridization or polymerase chain reaction (PCR)], or rearrangements of immunglobulin heavy-chain (*IgH*) or T-cell-receptor (TCR-β, –δ,–λ, and –γ) genes detected by PCR with a sensitivity of 10^{-3}–10^{-6} can be achieved (reviewed in ref. *69*). More recently, real-time PCR has been used for less cost intensive and more rapid MRD detection *(70)*.

For all methods of MRD detection, it is necessary to identify a patient-specific individual target—either a LAIP or a clonal rearrangement—at the time of diagnosis, which may be a logistical problem in some studies. From a theoretical point of view, MRD assessment is possible with either method in >90% of ALL patients. MRD evaluation may serve several purposes in adult ALL, as described in the following.

6.7.1. Redefinition of CR

In addition to CR definition according to conventional criteria, the term *molecular remission* should be added to treatment evaluation in ALL. From childhood ALL trials, it is evident that probably <50% of the patients achieve a molecular CR and that these patients have a favorable prognosis.

6.7.2. Definition of New Prognostic Factors

In childhood ALL it has been convincingly shown that positive MRD status is correlated with a poor outcome, whereas children without detectable MRD have a high chance of cure.

Table 6
Prognostic Relevance of MRD in ALL—
Current Knowledge and Open Questions

Time point	*MRD level (criterion)*	*Relapse risk (%)*
After induction	High (>10^{-3})	High (50–90%)
	Negative (<10^{-4})	Low (~10%)
	Intermediate (10^{-4}–10^{-3})	Unclear; follow-up required
During first year of treatment	Continously high (>10^{-4})	High (>70%)
	Decreased and repeatedly negative (10^{-4})	Low (~10%?)
	Fluctuating	Unclear; follow-up required
	Increased by >2 logs	Molecular relapse?
Before and after SCT	High (>10^{-3}–10^{-4})	High

Abbreviations: MRD, minimal residual disease; SCT, stem cell transplantation.

Based on data reported in refs. *71*, *74*, *75*, *78–82*.

This was demonstrated by flow cytometry *(71–73)* and by PCR detection of *IgH* and *TCR* rearrangements *(74–76)*. It seems to be established that patients with a high MRD level (>10^{-3}–10^{-4}) at any time after induction therapy have a higher risk of relapse (>40%) compared with patients with negative MRD status (<10%). Furthermore, MRD proved to be a prognostic factor independent of other "conventional" risk factors. Most importantly, by MRD evaluation, additional high-risk patients could be identified in "standard-risk" and "intermediate-risk" patients, as defined by conventional prognostic factors. Because of these findings, the German BFM group for childhood ALL has recently initiated a study with risk stratification based on conventional prognostic factors and on MRD status at wk 6 and mo 3 after induction therapy. In a recent analysis, this risk model was applied to intermediate-risk patients and identified subgroups with significantly different LFS rates of 45, 98, and 76% *(77)*.

In adult ALL only a few results on the prognostic value of MRD detection are available at present, but they generally confirm the results obtained in childhood ALL. In a small series Brisco et al. *(78)* demonstrated a high relapse rate (8/9) in patients with a high MRD level (>10^{-3}) after induction therapy. In patients with a low MRD level, the relapse risk was lower but still relevant (6/13), which may indicate that MRD kinetics are slower in adult patients compared with children. In a larger series of 57 adult ALL cases the incidence of MRD-positive results decreased rapidly (40, 38, 17, and 0% at 3-mo intervals from diagnosis) in patients remaining in continuous CR. During the first 2 mo from diagnosis, no correlation between relapse risk and MRD result was detected. However, the prognostic significance of MRD results increased stepwise, with the time being highest at 12–24 mo after diagnosis. Negative tests were more predictive for continuous CR than positive tests for relapse during the first 6 mo *(79)*.

The GMALL study group reported the results of a pilot study with MRD measurement based on *TCR* and *IgH* rearrangements. In 59 evaluable patients with at least one clonal marker and a sensitivity of <10^{-4}, a strong correlation between MRD level and relapse risk could be demonstrated. After 4 wk of induction therapy, relapses occurred in 3/5 patients with MRD >10^{-2} compared with 3/21 relapses in patients with MRD <10^{-2}. The prognostic relevance was even higher at later time points. Seventy-three percent (11/15) of patients with MRD >10^{-4} at one time point (mo 3 to mo 12 from diagnosis) relapsed, compared with only 13% (4/31) of the patients with MRD levels always below 10^{-4}. Based on these findings the GMALL has started a prospective study with MRD- based treatment decisions after 1 yr of chemotherapy in patients with standard-risk ALL according to conventional risk factors. For risk stratification according to MRD, the MRD level after induction therapy will be considered as well as the course of MRD during the first year. Patients with a high risk of relapse according to MRD will be transferred to treatment intensification (SCT or intensified maintenance) after 1 yr, whereas treatment will be stopped in patients with low risk of relapse. However, for all MRD-based treatment regimens, it has to be considered that patients may relapse despite negative MRD results, possibly because of the low sensitivity of the applied method, overly long intervals between evaluations, or clonal evolution of the leukemic blast cells. The prognostic relevance of MRD level and the course of MRD at different time points are summarized in Table 6.

MRD evaluation provides a unique option for individualization of treatment in ALL, avoiding undue toxicity in patients with a low risk of relapse and offering maximal therapy to patients with very high relapse risk. The ongoing studies for MRD-based risk stratification and treatment decisions will hopefully answer some of the outstanding questions in the field of MRD evaluation:

1. Technical requirements for MRD-based risk stratification (number of markers, sensitivity level?).
2. Optimal time point for MRD-based treatment decisions (3, 6 or 12 mo ?).
3. Subgroup-specific differences in the course of MRD and implications for treatment decisions.
4. Feasibility and outcome of MRD-based treatment decisions.
5. Molecular monitoring of single treatment elements (new consolidation cycles, antibody treatment, STI571, purging, donor lymphocyte infusions).
6. Options for MRD evaluation in peripheral blood.
7. Optimization of MRD-based risk stratification (reduction of intermediate-risk group).
8. Comparative analysis of different methods of MRD evaluation (PCR, real-time PCR, flow cytometry).

6.8. Drug Resistance and Drug Pharmakokinetics

Drug resistance may be an important factor for treatment failure in adult ALL. The overall resistance profile in individual patients can be evaluated with the methyl-thiazol-tetrazolium (MTT) assay from fresh or stored bone marrow or peripheral blood samples. In childhood ALL, it was shown that the results of MTT assays correlate with empiric experience on the efficacy of specific cytostatic drugs against certain subtypes of leukemia. Thus, a higher resistance was found in children older than 10 yr

Table 7
Prognostic Factors for Leukemia-Free Survival in Adult ALL

Category	*Low-risk features*	*High-risk features*
Age (yr)	Adolescence (15–20)	Older age (> 50 yrs)
WBC count (B-lineage)	<30,000/μL	> 30,000/μL
Immunophenotype	Thymic T-ALL	Pro-B, early T, and mature T
Cytogenetics and molecular genetics	Hyperdiploid karyotype (?) *TEL-AML1* (?)	(t(9;22)/*BCR-ABL* t(4;11)/*ALL1-AF4* Other cytogenetic/molecular aberrations (?)
Time to CR (wk)	CR < 2–4 wk	CR > 2–4 wk
MRD after induction	$< 10^{-3}$–10^{-4}	$> 10^{-3}$–10^{-4}
MRD during consolidation	$< 10^{-4}$ or negative	$> 10^{-4}$ or increasing
Other		In vitro drug resistance (?), *MDR* gene overexpression (?)

Abbreviations: WBC, white blood cell; CR, complete remission; MRD, minimal residual disease.

compared with younger children, in nonhyperdiploid ALL cells, and in relapsed childhood ALL; sensitivity to prednisone, L-asparaginase and vincristine (as major drugs of induction therapy) was associated with a better outcome *(83)*.

In adult ALL, only a few results of the MTT assay are available. In 43 retrospectively analyzed patient samples, a higher resistance to daunorubicin and prednisone, but not vincristine and L-asparaginase, was found in adult Ph/*BCR-ABL*-positive compared with negative ALL *(84)*, and a study evaluating in vitro resistance to prednisone showed an association with lower CR rates (66% vs 84%) and inferior LFS rates but not survival *(85)*. Recently, the Gruppo Italiano Malattie Ematologiche Maligue dell' Adulto (GIMEMA) also reported the prognostic relevance of multidrug resistance (MDR-1) expression *(86)*. Further analyses in adult ALL would be of interest to confirm in vitro drug resistance and MDR overexpression as additional risk factors, to describe resistance profiles in individual patients and distinct subgroups of adult ALL, and probably to evaluate options for modulation of MDR.

Optimization of chemotherapy also includes the measurement of drug sensitivity and individual differences in terms of drug pharmacokinetics and pharmacogenetics. In childhood ALL, polymorphisms in drug-metabolizing enzymes, transporter molecules, and targets of drug action are related to treatment efficacy, toxicity, and long-term treatment effects *(87)*. Thus, adjustment of MTX dosage to individual pharmakokinetics contributed to an improvement of clinical outcome in the St. Jude studies *(88)*. More pronounced hematologic toxicities were encountered in patients with mutations of the thiopurine methyltransferase locus, *(89)*, and these patients were also at higher risk for secondary brain tumors and acute myeloblastic leukemia. Similar investigations in adult ALL are lacking, although individualization of chemotherapy taking these differences into account may contribute to a further improvement of results and less toxicity in individual patients.

6.9. Risk Stratification in Adult ALL

In most large clinical trials there is agreement on the following risk factors for adult ALL: age, time to achieve CR, initial WBC count, cytogenetic and molecular aberrations (Ph/*BCR-ABL*, t(4;11)/*ALL1-AF4*). Other risk factors such as immunophenotype, MRD, and cytogenetic and molecular aberrations are not uniformly used in different studies (Table 7). Also, risk stratification does not always translate into risk-adapted treatment regimens. Furthermore, it has to be considered that prognostic factors are treatment-dependent and should therefore not be transferred from one study to another.

Since 1984 the GMALL group has devised risk-adapted treatment strategies with two major aims:

1. Allocation of patients to risk group-specific treatment schedules (e.g. short intensive treatment protocols for mature B-ALL) and the development of new subgroup-adjusted treatment regimens
2. Rational decisions on treatment intensity—particularly indications for SCT

7. TREATMENT OF PH/*BCR-ABL*-POSITIVE ALL

Ph/*BCR-ABL*-positive ALL is the subtype with the worst prognosis in children as well as adults. Remission rates have been increased to 75–80% in recent studies, but probably<10% of the patients achieve a molecular remission after induction therapy. Consequently, the remission duration with conventional chemotherapy is short (9 mo), and the long-term LFS rate in chemotherapy patients is <10%. Modifications of chemotherapy (e.g., inclusion of HDAC or HDMTX) did not lead to a significant improvement. At present the only chance of cure in Ph/*BCR-ABL*-positive ALL is SCT, although the results are again inferior compared with those for Ph/*BCR-ABL*-negative patients *(90)*. At present the best results are achieved with allo-SCT from sibling donors with an LFS rate of approximately 40%. In the largest series, with 33 and 24 patients, respectively, the LFS rates were 38 and 65% *(91,92)*. These considerable differences remain unexplained. Results of MUD-SCT in Ph/*BCR-ABL*-positive ALL are improving. In one recent series the LFS rate was 40% in adult patients with a "realistic" median age of 35 yr who were transplanted in first CR *(36)*.

The results of auto-SCT are inferior (25–30%) but still better than for chemotherapy alone. In the largest series, 23 patients, the LFS rate was 25% at >2 yr, giving a realistic picture of what can be achieved with auto-SCT in Ph/*BCR-ABL*-positive ALL *(93)*. In the latter series it could be demon-

strated that purging with immunomagnetic beads and antibodies reduced the tumor load in stem cell grafts by 2–3 logs. Furthermore, it was shown that the content of *BCR-ABL*-positive cells is significantly lower in peripheral stem cell compared with bone marrow grafts *(94)*. Since SCT is increasingly included in the treatment of Ph/*BCR-ABL*-positive ALL, overall survival has improved slightly. One major problem is that this disease occurs in an older patient population with limited options for SCT and other types of chemotherapy intensification.

Therefore, "nonchemotherapy" approaches are of particular interest. Furthermore, in this disease subtype the causal molecular mechanisms are rather well described and include an upregulation of tyrosine kinase activity induced by the *BCR-ABL* fusion gene product. Several "causal" treatment approaches directed to the selective suppression of the *BCR-ABL* gene and its gene products have been discussed in the past. They include antisense oligonucleotides, ribozymes, ex vivo generation of cytotoxic T-lymphocytes, and others. The most promising approach is the selective inhibition of the *ABL*-tyrosine kinase with STI571. This is also the first molecular treatment approach that has shown significant clinical efficacy in larger patient numbers. Cellular proliferation of *BCR-ABL*-positive chronic myeloid leukemia and ALL cells could be inhibited selectively *(95)*. Promising results have also been achieved with this agent in a phase II study in heavily pretreated patients with relapsed or refractory Ph/*BCR-ABL*-positive ALL, with hematologic responses observed in 19 of 32 patients *(96)*. Furthermore, clinical responses were correlated with MRD levels in bone marrow and peripheral blood *(97)*. Thus, quantitative PCR provides an option for continuous monitoring of the therapeutic effects of STI571. Oral treatment with STI571 is generally well tolerated and also feasible in elderly patients. Based on these promising results, phase II studies in patients with *de novo* Ph/*BCR-ABL* positive-ALL have begun. Thus, the GMALL group has initiated a trial with application of STI571 after induction therapy and after SCT in patients with MRD. It can already be anticipated that single-drug treatment with STI571 will not lead to cure in most patients with Ph/*BCR-ABL*-positive ALL. Therefore, clinical trials of this agent combined with other chemotherapy and with molecular approaches (e.g., farnesyl transferase inhibitors) are of great interest.

8. FUTURE PROSPECTS

The optimization of chemotherapy, stem cell transplantation, supportive care, and the exploration of new treatment approaches will hopefully lead to further improvement in the outcome for adult ALL patients. These strategies include:

1. Refined subgroup-adjusted treatment, as already suggested for T-ALL (Cp, ara-C), pro-B-ALL (HDAC), B-precursor ALL (HDMTX, 6-MP), or mature B-ALL (HDMTX, HDAC).
2. Extended indications for SCT and inclusion of new modalities such as better conditioning regimens, NMSCT, utilization of GvL effects (e.g., by donor lymphocyte infusions).
3. Molecular treatment approaches (e.g., *ABL*-tyrosine kinase inhibitors).
4. Evaluation of drug resistance and methods for MDR modulation.
5. Evaluation of drug pharmacokinetics and pharmacogenomics for individualized treatment adaptations with higher efficacy and lower toxicity.
6. Prospective evaluation of MRD, development and confirmation of MRD-based risk stratification, and treatment adaptation.
7. Evaluation of immunotherapy for B- as well as T-lineage ALL.

Taken together, these new treatment options seem promising as means to improve clinical outcome in adult ALL, above the level achieved in the last two decades with chemotherapy alone. However, well-designed studies and probably joint efforts are needed to explore optimal combinations, timing, and dosage of conventional and new treatment approaches.

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CHEMOTHERAPEUTIC STRATEGIES

II

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CENTRAL NERVOUS SYSTEM THERAPY FOR ACUTE LYMPHOBLASTIC LEUKEMIA

11 Central Nervous System–Directed Therapy for Acute Lymphoblastic Leukemia

JUDITH M. CHESSELLS

CONTENTS

1. INTRODUCTION

Over 30 years have elapsed since the first description of leukemic infiltration of the central nervous system (CNS) in children with acute lymphoblastic leukemia (ALL) *(1)*. As patients treated in the 1960s and 1970s achieved longer hematologic remissions, CNS relapse developed in up to 80% of children remaining in bone marrow remission *(2)*. Children with a high initial leukocyte count were at risk of early CNS disease *(3)*, but it was not possible to identify a group with no risk of this complication.

Although rapid control of overt CNS relapse could be achieved in the vast majority of children with a course of weekly intrathecal methotrexate (IT MTX), the disease was difficult to eradicate, caused chronic symptoms, and was almost inevitably followed by a later bone marrow relapse *(4,5)*. Moreover, patients with chronic CNS disease were at increased risk of severe leukoencephalopathy, progressive neurologic deterioration and dementia associated with characteristic white matter changes visible on computed tomography (CT) scan *(6)*.

The histopathologic changes in children with chronic CNS disease show a characteristic pattern *(7)*. Briefly, leukemic cells in the walls of the superficial arachnoid veins, which may proliferate slowly and presumably are present at the time of diagnosis, infiltrate and destroy the arachnoid trabeculae, with penetration of the channels for cerebrospinal fluid (CSF) circulation *(8)*. Thus, CNS leukemia is largely a leptomeningeal disease, with progressive parenchymal involvement occurring at a later stage. These findings have two implications: first, subclinical CNS infiltration is probably present in all patients, and second, IT treatment is likely to be effective against early or presymptomatic disease. Many years were to elapse before the significance of the latter implication was fully recognized.

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2. CURRENT STATE OF THE ART

2.1. Evolution of CNS-Directed Therapy

The early 1970s witnessed the introduction of CNS-directed therapy, often misleadingly called "CNS prophylaxis." Results of a number of prospective randomized trials conducted by St. Jude Children's Research Hospital (SJCRH), the American Children's Cancer Group (CCG), and the UK Medical Research Council ALL trials (MRC UKALL) showed that children receiving presymptomatic CNS-directed therapy had superior event-free survival (EFS), overall survival (OS), and lower morbidity than did those who were treated only after the development of overt disease *(9–11)*. Since radiotherapy had previously proved effective in control of overt CNS disease, these early trials included irradiation. Effective CNS-directed therapy could be provided by whole neuraxis irradiation in a dose of order of 24 Gy *(9,11)*, or a combination of 24 Gy of cranial irradiation and a course of IT MTX injections *(12,13)*, but not by a course of short-term IT MTX alone *(14)*. Craniospinal irradiation was more myelosuppressive than cranial irradiation and IT MTX, and it compromised systemic therapy. Thus, 24 Gy cranial of irradiation, subsequently reduced to 18 Gy as a result of a comparative study by the CCG *(15)*, and a course of five or six IT MTX injections, given early during therapy at a dose calculated according to age *(16)*, became the norm for CNS-directed therapy in many protocols developed during the following 15–20 yr. Although the results of treatment of adult ALL were and are much inferior to those in children *(17)*, this approach was also introduced into many adult

protocols, with or without additional intensive treatments, such as high-dose cytarabine *(18)*.

There were a few dissenters from this approach. The American Pediatric Oncology Group (POG) had shown in a randomized trial conducted in the 1970s that regular IT chemotherapy with the combination of hydrocortisone, cytarabine, and MTX, the so-called triple IT therapy (TIT), was as effective as the combination of cranial irradiation and IT MTX in preventing CNS relapse *(19)*, and the group continued to use TIT for CNS-directed therapy thereafter, except for children with T-cell ALL. The Norwegians tended to avoid cranial irradiation in most patients and were early users of high-dose intravenous MTX *(20)*; they were later joined by the rest of the Scandinavian countries.

Two important randomized trials from this era continue to provide lessons for the modern management of ALL. A classic study from the old Cancer and Acute Leukemia Group B (CALGB), with recently updated results, demonstrated the "swings and roundabouts" interaction between systemic and CNS-directed therapy. Between 1976 and 1979, CALGB randomized 596 children and adolescents to receive intermediate-dose MTX and regular IT MTX, or cranial irradiation and IT MTX, all other aspects of treatment being identical *(21)*. Intermediate-dose MTX afforded better testicular and systemic protection and cranial irradiation better CNS protection, but the overall EFS at 11 yr, although inferior to that obtained with modern therapy, remained similar in both groups *(22)*. There is experimental evidence that dexamethasone is more effective than prednisolone *(23,24)*, and a second late publication from the CALGB, reporting on a randomized trial conducted in the early 1970s, showed that the CNS relapse rate was significantly lower in patients randomized to receive oral dexamethasone during induction than in those given prednisone *(25)*.

2.2. Recognition of the Late Effects of Treatment

As increasing numbers of children with ALL achieved long-term survival, it became apparent that, although most children are well and symptom-free, a sizeable minority have adverse late effects of therapy. A retrospective survey of children treated at the Hospital for Sick Children (Great Ormond Street, London) in the era of cranial irradiation and IT MTX showed that about one-third of children alive in first remission had significant problems, which were either neuropsychological or involved growth and puberty *(26)*. Needless to say, all complications were more prevalent and severe in patients who had relapsed and survived after retrieval therapy. More recently, we reviewed the outcome for children surviving after relapse who had received a second course of irradiation; we found growth hormone deficiency in all and significant neuropsychological impairment in 12 of the 14 children tested. Girls and younger children were more at risk for problems *(27)*.

A wide range of neurologic and psychological problems has been noted in long-term survivors of ALL who remain in first remission, particularly children, who are more vulnerable and more numerous than adults. The most severe complication, MTX-radiation encephalopathy *(28)*, is seen largely in patients with recurrent CNS disease *(6)*. It has normally been observed only in survivors in first remission who have received the combination of cranial irradiation, IT MTX, and parenteral MTX *(29)*. However, long-term survivors in first remission have developed a variety of learning difficulties and problems with memory and concentration; these may or may not be associated with the changes in white matter and calcification seen on CT scans or magnetic resonance imaging (MRI). There is now a large literature on this topic, and the reported frequency of such problems is variable *(30)*, although girls *(31–33)* and younger children *(33,34)* are more vulnerable. Adults seem to be at less risk for toxicity, at least after the radiation doses used in CNS-directed therapy *(35)*, although it is possible that the problems in adults are underestimated *(36)*.

These neuropsychological sequelae have been, at least in part, ascribed to cranial irradiation. Comparative studies of children surviving ALL and brain tumors seemed to show, as expected, a relationship between the dose of irradiation and the degree of neuropsychological impairment *(37)*. This finding gave additional impetus to reduction of the radiation dose from 24 to 18 Gy, a measure still associated with deterioration in neurocognitive function *(38,39)*.

Long-term follow-up of the CALGB trial comparing cranial irradiation and iv MTX showed that children who had received cranial irradiation had significantly worse academic achievement, self-image, and psychological distress than did those who had been treated with iv MTX *(40)*. On the other hand, prospective neuropsychological follow-up of patients who received either cranial irradiation (24 or 18 Gy) or repeated iv MTX infusions in St. Jude's Total Therapy Study X, showed no difference between groups with respect to any neuropsychological outcome measure *(41)*, while patients who had received either 18 Gy or iv MTX showed abnormalities on electroencephalograms (EEGs) and CT scans *(42)*. The picture is a complex one, and the risk of toxicity after cranial irradiation may be influenced by other treatment variables, such as administration of IT MTX *(43)* or iv MTX *(44)* before irradiation.

The evidence that cranial irradiation is associated with an increased risk of brain tumors is incontrovertible. A review of patients treated by the CCG between 1972 and 1988 showed that, among over 9000 long-term survivors of ALL, there was a 22-fold increase of second neoplasms in the CNS, all of which occurred in children who had received cranial irradiation. Children under 5 yr of age at diagnosis were particularly vulnerable *(45)*. Cranial irradiation and young age have both been identified as risk factors for secondary CNS tumors in other, smaller series of patients *(46,47)*, and it has been suggested that intensive antimetabolite therapy before and during radiotherapy is an additional risk factor *(48)*.

Cranial irradiation is also associated with the risk of premature or precocious puberty, particularly in girls *(49)*, and this complication (when accompanied by a degree of growth hormone insufficiency) can cause significant reduction in final height. Growth hormone insufficiency may require intervention *(50)* and may be associated with the development of osteoporosis *(51)*.

2.3. Current Strategies for CNS directed therapy

Concerns about the potential late effects of treatment in the growing child have recently led to omission of cranial irradia-

tion in some protocols and dose reductions in others. Despite the many thousands of patients who have been enrolled in trials of CNS- directed therapy, there is still no consensus about the best approach to treatment. The obstacles to agreement include the wide diversity of systemic treatments, which themselves have CNS effects, and the happily low number of adverse CNS events associated with modern treatment regimens. With published EFS rates for pediatric ALL nudging 80% in some trials *(52)*, at least in the developed world, trials that focus on CNS protection *per se* are not feasible without very large numbers of patients. The present emphasis, appropriately, is directed toward increasing overall EFS rates and minimizing the late effects of treatment.

Table 1 summarizes the strategies for CNS protection that have been incorporated into some recently published pediatric trials. No details are given for systemic therapy, which of course also influences CNS remission. The information is derived from the publications cited and is incomplete in some instances. This list, which is by no means comprehensive, indicates the wide diversity of current approaches to CNS protection and hence the difficulties in reaching any consensus.

The CCG demonstrated in a series of randomized trials that, provided patients received appropriately intensive systemic therapy, adequate CNS protection could be achieved by giving IT MTX during induction and intensification and regular IT MTX during continuing treatment in both low-risk *(53)* and intermediate-risk *(54)* groups. Cranial irradiation in the CCG 1800 series was given to patients with the lymphoma/leukemia syndrome and to high-risk children and adolescents who had a poor response to early induction therapy as shown by a high proportion of bone marrow blasts on d 7 of therapy. Those high-risk children with a good response to induction therapy were randomized to receive 18 Gy of cranial irradiation or intensified IT MTX. Although interim analysis suggested that the arm including cranial irradiation was superior, longer follow-up showed no significant difference between the two arms and fewer late events in children treated with MTX alone *(55)*. The CCG relies on intensified systemic and IT therapy, largely with MTX but some cytarabine and no high-dose iv MTX. Few patients are irradiated.

An early randomized trial conducted by the Berlin–Frankfurt–Münster (BFM) group compared intermediate-dose iv MTX (0.5 g) and cranial irradiation (18 Gy) in standard-risk children with ALL. The CNS relapse rate and overall EFS were inferior in patients treated with MTX *(56)*. The group has since continued to use cranial irradiation for all except the lowest-risk patients, although they have decreased the radiation dose to 12 Gy and irradiation is preceded by infusions of MTX in a dose now increased to 5 g. The patients do not receive IT therapy during continuing treatment *(57)*. These modifications have tended to be based on comparisons with historical controls rather than randomization. Other disciples of the BFM group, such as the Dutch *(58)* and the Italians *(59)*, have adopted BFM-style protocols but have abandoned cranial irradiation, at least in all except the highest-risk patients, with substitution of more intensive and longer term IT chemotherapy.

The MRC UKALL XI trial (1990–1997) randomized children with leukocyte counts < 50×10^9/L to receive regular IT MTX with or without additional high-dose iv MTX and those with higher counts to cranial irradiation or iv/IT MTX *(60)*. The successor protocol relies on IT MTX alone for standard-risk patients. POG continues to advocate cranial irradiation for higher-risk T-ALL patients *(61)* and have for many years used TIT for all patients with B-progenitor cell ALL *(62)*.

The overall consensus from these studies is that most collaborative groups have either abandoned cranial irradiation in a large proportion of children or significantly reduced the dose. High-dose MTX remains fashionable, and there are various schemes and doses for administration. Most protocols without cranial irradiation continue to specify regular IT chemotherapy during continuing (maintenance) treatment.

3. PROBLEMATIC ISSUES IN CNS-DIRECTED THERAPY

3.1. The Diagnosis of CNS Disease

The classical criteria for diagnosis of CNS disease are a CSF pleocytosis of >5 cells/mm^3 and the presence of recognizable blast cells on a well-stained cytospin preparation. This concept, which has the advantage of simplicity and was agreed on at the Rome meeting addressing the staging of ALL *(63)*, has been somewhat complicated by the introduction of the "borderline," or CNS2, CSF status. Workers at SJCRH have reported that patients with <5 cells but recognizable blasts in the CSF at diagnosis (so-called CNS2) are at an increased risk of CNS relapse *(64)*.

At a subsequent meeting to discuss problems in risk assignment in ALL, it was suggested that the definition of CNS involvement should be refined, with CNS1 indicating a clear CSF, CNS2 a low number of blasts, and CNS3 unequivocal CNS involvement *(65)*. The significance of these distinctions remains controversial. Workers at SJCRH have claimed that the adverse significance of CNS2 can be obviated by early intensive IT therapy *(66)*. The CCG have failed to confirm the significance of CNS2 disease at diagnosis *(67)*. This discrepancy may reflect differences in systemic and CNS-directed therapy between the groups.

Similar problems may arise with the interpretation of CSF pleocytosis or apparently abnormal cells seen on cytospin preparations during treatment. For example, a small series of patients from SJCRH with apparent CNS relapse achieved a remarkable 70% EFS at 5 yr *(68)*, but several of these patients could be classed as CNS2. Thus, the appearance of blast cells in CSF samples with a low cell count during treatment has been predictive of relapse in some studies *(69)* but not others *(70)*.

The distinction between leukemic blasts and reactive mononuclear cells is not always easy, and standard morphologic studies have been supplemented with immunocytochemical *(71,72)* and newer molecular techniques. These investigations may be helpful, particularly in cases with pleocytosis and difficult morphologic interpretations. Their use to identify low numbers of blast cells and thus "upstage" patients at diagnosis or justify early intervention and possible radiation during treatment needs careful consideration.

Now that most patients receive regular IT chemotherapy, the diagnosis of CNS relapse is often made on the basis of a routine therapeutic lumbar puncture. Repeat lumbar puncture

Table 1
Recently Published Strategies for CNS Protection in ALL

			CNS-directed treatment				
			IT				
Group	*Protocol*	*Systemic therapy*	*Early*	*Ongoing*[a]	*IV MTX*	*Cranial irradiation*	*Comments*
AEIOP *(59)*	ALL 88 (88–92)	Risk-adapted BFM type	MTX	MTX	5.0 g/m^2 4 courses	None except for high-risk group	Protocol for intermediate- and lower risk children; nonrandomized study
BFM *(57)*	BFM 90 (90–95)	Risk adapted	MTX	No	5.0 g/m^2 4-6 courses	12 Gy	No IT therapy during maintenance; TIT for highest risk group; nonrandomized study
CCG *(55)* [b]	1882 (89–95)	High-risk patients	MTX	MTX	No	Nonrandomized for nonresponders	Randomization of good responders to cranial irradiation.
DFCI *(114)*	87-01 (87–92)	Risk adapted	Both	Both	Yes	18 Gy, higher risk group	MTX and cytarabine given IT
Dutch *(58)*	ALL-7 (88–91)	Intensive BFM type	MTX	No	5.0 g/m^2	Only if CNS involved	Protocol based on BFM-86 but no radiation
MRC UKALL *(95)* [b]	UKALL XI (90–97)	Two vs three intensifications	MTX	MTX	6–8 g/m^2 3 courses	Randomized to 24 Gy, higher risk group	24 gy vs IV MTX in high-risk group, iv vs IT alone in lower-risk group
SJCRH *(66)*	Total XIII (91–94)	Risk-adapted IT and systemic	TIT	Varied	MTX	18 Gy, higher risk group	Delayed cranial irradiation
POG *(61,115)*	8602 (86–91)	Antimetabolite based	TIT	TIT	1 g/m^2 × 6	None	Standard-risk patients with pro-B-cell ALL
	8704 (97–92)	Intensive multi-agent	TIT	TIT	No	24 Gy for cases with WBC >50 × 10^9/L	Protocol for T-cell ALL only

Abbreviations: IT, intrathecal; TIT, triple intrathecal therapy: MTX, methotrexate; CNS, central nervous system; AIEOP, Italian Association for Pediatric Hematology and Oncology; BFM, Berlin–Frankfurt–Münster Group; CCG, Children's Cancer Group; DFCI, Dana-Fraber Cancer Institute; MRC, Medical Research Council; SJCRH, St. Jude's Children's Research Hospital; POG, Pediatric Oncology Group.

[a] IT therapy during continuation (remission maintenance) treatment.

[b] Randomizations to CNS treatment.

in 2–3 wk is desirable in borderline cases, but the diagnosis may be masked because of effects of the recent IT therapy.

The diagnosis of overt CNS disease in the absence of CSF infiltration is difficult. Most patients with cranial nerve palsies or hypothalamic syndrome have blasts in the CSF, and these clinical features are accepted as indicative of CNS disease. Other neurologic symptoms, particularly convulsions, are more likely to be symptomatic of toxicity than relapse. CT is usually not helpful in the diagnosis of CNS disease in ALL, except in very rare cases with focal lesions, and reported experience with MRI is limited. A rare problem, usually occurring soon after treatment is stopped, is an ocular relapse. This situation presents with unilateral iridocyclitis but often without CSF infiltration *(73,74)*. Diagnosis can be confirmed by finding leukemic cells in the anterior chamber or by biopsy of the iris.

Despite the availability of more tests for diagnosis of overt CNS relapse, there seems to be little practical clinical benefit from such additional investigations, except perhaps to confirm CNS infiltration in cases with large numbers of cells and difficult morphologic distinctions. The concept of CNS2 will be difficult to evaluate, as most patients already receive intensive IT and systemic treatments. Ideally, the findings from SJCRH will be tested in a large blinded prospective study.

The diagnosis of CNS infiltration either at diagnosis or at relapse, with its implications of intensified treatment and probable radiation therapy, needs a firm basis. It is normally our practice to perform a diagnostic lumbar puncture at the same time as the diagnostic bone marrow testing in newly diagnosed children with leukemia. Both procedures are invariably performed under a general anesthetic. If the diagnosis is already obvious from the blood film and clear CSF is obtained, then IT chemotherapy is given at the same time. If the diagnosis is in doubt or there are difficulties with the lumbar puncture, chemotherapy is not given, and a repeat lumbar puncture is performed in the next day or so.

CNS relapses, unlike marrow relapses, tend to occur in the first 2–3 yr from diagnosis *(75–77)*. There is no justification to continue surveillance lumbar punctures in children who have completed treatment, but the onset of headaches or other symptoms warrant prompt examination of the CSF.

3.2. Management of Patients with CNS Disease at Diagnosis

Assuming sufficiently sensitive tests were available, virtually all patients would have subclinical evidence of CNS disease at diagnosis. With conventional criteria, 2–3% *(57,78,79)* of children have overt CNS disease at diagnosis, whereas perhaps another 2% have a bloody tap or CNS2 infiltration . There is no clear evidence about the best treatment of these latter cases, and an argument can be made for continuing the agreed-on standard treatment.

Patients with unequivocal CNS disease at diagnosis used to be deemed high risk, and most protocols have separate treatment recommendations for this group. The CCG have reviewed the results of management of such patients and recently concluded that when they are given intensified therapy and craniospinal irradiation, the outlook is comparable to that of other patients. Cranial irradiation is given at a dose of 24 Gy, and spinal irradiation has been empirically reduced from 12 Gy to 6 Gy *(78)*. The BFM group has given appropriate risk-adapted systemic therapy, additional IT therapy, and 24 Gy of cranial irradiation *(57)* with no significant difference in EFS between these and other patients; the SJRCH gave 24 Gy of cranial irradiation to patients with overt CNS disease.

These data are all derived from small numbers of patients and comparisons of modifications of treatment with historical controls, but there seems no real evidence to support the use of spinal irradiation in patients with CNS disease at diagnosis; a combination of aggressive IT and systemic chemotherapy with cranial irradiation seems to be appropriate. The case for continuing cranial irradiation seems established, although effective CNS control has been demonstrated in some groups of patients, most notably infants with ALL *(80)*, and possibly patients with B-non-Hodgkin's lymphoma/ALL and CNS involvement, in whom encouraging results have been reported with high-dose systemic and intraventricular therapy *(81)*.

With CNS disease at diagnosis being relatively rare, and satisfactory results being obtained with present treatment, the scope for clinical trials is limited. There would seem to be sufficient evidence to abandon the use of spinal irradiation in this situation. It seems possible that some patients can be cured without cranial irradiation, but the toxicity and expense of alternative high-dose therapies might outweigh the theoretical benefits, at least in patients with standard-risk ALL.

3.3. CNS-Directed Therapy in Adults

Protocols for the treatment of adult ALL have highly selective entry requirements and poor overall results, particularly when results are directly compared with those in children treated on similar protocols *(82)*. A comparative study from the United Kingdom, in which both adults and children received a course of IT MTX, cranial irradiation, and similar chemotherapy, illustrated that the adults had significantly more marrow relapses and failures to achieve remission. There was no difference in CNS relapse rate. The overall poor results in adult ALL have meant that questions about CNS protection have not been prominently featured in randomized trials.

The German group has some of the most successful reported results in adult ALL and has long used a risk-adapted strategy *(83)*, as have their pediatric counterparts. The patients in these studies have largely received cranial irradiation and IT MTX, but with increasing emphasis on intensified systemic therapy. A retrospective review of serial protocols from the M.D. Anderson Cancer Center showed that with increasing intensity of systemic and IT chemotherapy, CNS relapse rates were reduced from 31 to 3% without recourse to cranial irradiation *(84)*.

A recent comprehensive review of CNS-directed therapy in adult ALL *(85)* confirmed the need for adequate CNS protection for all age groups. This objective can be achieved with early and continuing IT therapy in combination with high-dose chemotherapy and/or cranial irradiation. Adults, like children who sustain a CNS relapse, have a poor prognosis and are at high risk of subsequent marrow relapse.

Continued efforts to improve outcome in adult ALL will probably focus on the stratification of patients according to the

biology of their disease and the evaluation of intensified therapy with stem cell rescue. The major objective must be improvement in overall EFS.

3.4. The Role of Cranial Irradiation in CNS-directed Therapy

It is clear from Table 1 that the place of cranial irradiation for children with newly diagnosed ALL and no evidence of CNS disease at diagnosis has significantly diminished. Are there any children in this category for whom cranial irradiation is of established benefit? This question is hard to address because of the paucity of randomized trials exploring this issue and the small number of CNS events.

Patients with T-ALL have been historically deemed a group at high risk for CNS relapse. A retrospective comparison was made between T-cell ALL patients with a good response to steroids treated on the BFM 90 and the Italian ALL 91 studies. The protocols were very similar except that BFM patients received 12 Gy of cranial irradiation and IT MTX, and the Italian patients got TIT. The BFM patients had a 3-yr EFS that was similar in patients with an initial leukocyte count of less or more than 100×10^9/L. The Italian results were significantly worse in patients with a high leukocyte count, 80.6% vs 18%. However, these results were obtained retrospectively and were based on a very small number of patients *(86)*.

The CCG 123 protocol for lymphoma/leukemia (an entity comprising most patients with T-cell ALL) included a comparison between cranial irradiation and regular IT chemotherapy for children treated according to the old LSA2L2 protocol. The results showed that cranial irradiation was beneficial *(87)*, at least for patients with a leukocyte count $>50 \times 10^9$/L. However, the overall EFS in this arm was significantly inferior to the two more intensive arms, both of which included cranial irradiation *(88)*. Subsequent intensification of therapy in the next generation of protocols improved EFS *(89)*.

In UKALL XI and in ALL97, the successor trial, the UK MRC incorporated a randomized comparison of three high-dose MTX infusions and continuing regular IT MTX and 24 Gy of cranial irradiation with short-term IT MTX for children with an initial leukocyte count $>50 \times 10^9$/L. Both parents and participating physicians have found randomization difficult because of perceived problems after cranial irradiation.

The question of whether there is any group of patients with ALL who truly benefit from cranial irradiation is an important and emotion-laden one that, despite the need for large-scale collaboration, could be answered in prospective randomized trials. If there is a role for radiation in CNS-directed therapy, it would seem to be in patients with a poor response to induction therapy and/or those with a high initial leukocyte count and/or T-cell ALL. It seems most likely that this question will be resolved by gradual sequential reduction of the dose of cranial irradiation, as in the BFM studies, or by trial and error as groups arbitrarily decide to abandon it for alternative treatments. An important component of trials of primary CNS-directed therapy should be prospective neuropsychological assessment. Unfortunately, this is frequently missing because of expense, need for very long-term follow-up, and attrition of patients, particularly those in the higher-risk groups. Alternatives to cranial irradiation, although less likely to cause brain tumors or growth failure, may prove to have appreciable neurotoxicity.

3.5. Modifications of IT Therapy

As shown in Table 1, different collaborative groups have generally, tended to rely on the use of MTX alone, usually in a dose adjusted for age *(16)*, or on TIT, although some protocols incorporate a combination of the two or a few additional injections of single-agent cytarabine. There have never been any direct comparisons of the efficacy of IT MTX and cytarabine in the treatment of ALL, although one randomized trial comparing MTX with TIT is in progress.

The POG is the most confirmed users of TIT, but in 1995 this group reported that a pilot study involving IT MTX in combination with oral MTX and iv 6-mercaptopurine (for intensification) resulted in an unexpectedly high incidence of CNS and bone marrow relapses *(90)*. This led POG to switch from IT MTX to TIT for all patients in an ongoing large randomized trial comparing various schedules of iv 6-mercaptopurine and MTX for consolidation. A variety of neurologic complications including seizures and weakness associated with abnormalities on CT scans or MRI were subsequently reported in patients receiving TIT, more frequently when given in combination with iv MTX. The two arms of the protocol, which included 12 courses of iv MTX given fortnightly, carried the highest risk of encephalopathy. Retrospective comparisons also suggested that the switch from MTX to TIT was associated with an inferior overall EFS *(91)*.

There is at present no direct evidence that TIT is superior to IT MTX alone, but the variety of systemic therapies given to patients makes it very difficult to determine the benefits of any specific component of therapy. The results of randomized comparisons will be of interest. The search for intensification regimens that rely on antimetabolites is based on a commendable desire to minimize late effects of treatment, but the report from POG *(91)* serves as a salutary reminder that removal of cranial irradiation does not obviate all risk of neurotoxicity.

3.6. Modifications of Systemic Therapy

A recent nonrandomized Dutch study, using dexamethasone during induction and continuation treatment in combination with antimetabolite-based therapy, produced an excellent EFS with a CNS relapse rate of <2% in children with average-risk ALL *(92)*. This simple modification of standard treatment is undergoing evaluation in further randomized trials.

It is clear that adequate CNS protection can be afforded to most patients with systemic chemotherapy and IT chemotherapy without recourse to iv MTX, although the latter is effective in the treatment of established CNS disease *(93)* and is a component of many protocols. It is virtually impossible to evaluate the relative merits of, say, four infusions of MTX at 5 g/m^2 with modest dose cranial irradiation (the BFM approach) and continuing IT MTX with no infusions of MTX or cranial irradiation (the CCG approach), as both treatments are effective for most patients.

Two randomized trials have compared standard therapy with IT MTX and additional iv MTX. The CCG 139 protocol randomized 164 standard-risk patients to receive conventional

therapy and regular IT MTX or additional iv MTX 0.5 g/m^2 during consolidation and continuation therapy. There was no difference in EFS or distribution of relapses between the two regimens *(94)*. MRC UKALL XI had a similar design; >1500 children were randomized to receive iv MTX (6–8 g/m^2) for three doses every 2 wk during consolidation. Patients receiving the high-dose MTX arm had a significantly lower CNS relapse rate but similar overall EFS *(95)*. There is no evidence that moderate- or high-dose iv MTX is essential for the treatment of lymphoblastic leukemia, except probably in B-cell ALL, in which it is a component of most successful protocols *(96)*.

The bewildering variety of dose and rescue schedules precludes evaluation of any "best dose" or "best regimen" for iv MTX if it is to be used. Neurotoxicity is clearly schedule-dependent. The same caveats apply to iv administration of high-dose etoposide *(97)* and cytarabine *(98)*, which may have a role in selected protocols.

It is difficult to see how the role of MTX infusions can be properly evaluated. Despite its massive use throughout the world, this treatment has received little systematic prospective randomized evaluation. The potential efficacy of high-dose MTX therapy may be compromised by noncompliance with dose schedule or "overrescue" with folinic acid *(99)*, an issue that further complicates efforts to compare results.

3.7. Management of CNS Relapse

Despite intensive CNS-directed and systemic therapy, as many as 5–10% of children may develop a CNS relapse as a first event. Table 2 lists some published overall survival figures for such patients, all of whom were treated in large studies and had received primary CNS-directed therapy. The results show first that the outlook after CNS relapse is poor and second that the time to relapse is the most important factor for predicting outcome. Boys may be at higher risk of CNS relapse and may also have a worse prognosis thereafter *(100)*. Patients in the UK study *(75)* had all received cranial irradiation during first-line therapy. The report from the CCG 100 series of trials *(76)* contains a mixed group of patients, many of whom had not received prior cranial irradiation. Analysis of survival after relapse showed that patients who had received prior irradiation had a worse survival at 6 yr, although the difference was not statistically significant. Table 2 also shows some survival figures for children who relapsed in both the CNS and other sites.

Children with an apparent isolated CNS relapse normally have molecular evidence of occult marrow disease *(101,102)* and are at high risk of subsequent marrow relapse *(5,103)*. Further induction and systemic intensification of treatment are clearly as important as control of CNS disease. However, the best overall approach to management is uncertain. The low number of CNS relapses means that any randomized trials of therapy will either involve small numbers of patients or require widespread, possibly international collaboration. Moreover, many published reports about the management of CNS relapse emanate from an era when patients had received cranial irradiation as first-line therapy. A further complication, as discussed above, lies in the lack of an agreed-on definition of CNS leukaemia—a higher threshold for definition naturally including patients more likely to do well.

Table 3 shows some reported results of treatment for CNS relapse in recent publications. (The degree of selection of patients is unclear.) The treatment regimens vary widely, but most incorporate both intensified systemic and IT chemotherapy. An early randomized trial from the POG compared cranial and craniospinal radiotherapy and found no difference in CNS relapse rate between the groups *(104)*. The large trial by Winick and colleagues *(105)* from the POG compared two regimens for systemic therapy, with no significant difference in outcome between them. The most recent report involving sizeable numbers of patients, also from the POG *(106)*, used the accepted definition of CNS relapse. Most of the patients had previously received antimetabolite-based therapy, and none had received previous cranial irradiation. The protocol included high-dose etoposide, cytarabine, and MTX, as well as 24 Gy of irradiation to the cranium and 15 Gy to the spine given at 6 mo from relapse. The 4-yr EFS for patients whose initial remission was <18 mo was 46.2%, compared with 83.3% for those with a remission of >18 mo.

It would be a brave person who would omit cranial irradiation in children with overt CNS relapse, but there does seem to be evidence that irradiation can be delayed to allow a period of intensification. Is the spinal component of CNS irradiation, which produces short-term myelosuppression and long-term spinal shortening, really essential? The frequently cited justification for spinal irradiation lies in a tiny but still-quoted randomized trial conducted almost 30 yr ago by the UK MRC in children with a first relapse of ALL in the CNS. All children received IT MTX to clear the CSF and were randomized to receive craniospinal irradiation or cranial irradiation: no further IT therapy was given. All eight patients receiving cranial irradiation had a second CNS relapse, but only two of nine treated with craniospinal irradiation did so *(107)*. However, critical evaluation of more recently published data suggests that a combination of long-term regular IT chemotherapy and cranial irradiation may be as effective as craniospinal irradiation in preventing further relapse. This policy has been adopted in the United Kingdom for several years without apparent loss of CNS control.

Is there a role for administration of chemotherapy via an intraventricular (Ommaya) reservoir? This route, particularly in children who have undergone repeated lumbar punctures, may produce more consistent CSF levels of drugs *(108)* and, in multiply relapsed patients, longer CNS remissions than injections by the lumbar route *(109)*. Intraventricular MTX is, however, more neurotoxic, and although a few workers have attempted to evaluate a combination of intraventricular drugs and low-dose irradiation in second remission *(110)*, most would reserve this for patients with recurrent episodes of CNS disease in order to achieve symptom control.

Is there a role for high-dose chemoradiotherapy and autologous or allogeneic bone marrow transplantation (ABMT or BMT, respectively) in the management of CNS relapse? A small retrospective comparison between chemotherapy and ABMT or BMT from the BFM group showed no significant difference between the results of the two types of transplantation; both had a worse result than conventional treatment *(111)*. A small single-arm study from Italy suggested that

Table 2
Survival in Second Remission in Children with ALL and CNS Relapse

				Early Relapse			*Intermediate Relapse*			*Late Relapse*		
Author	*Primary treatment*	*Follow-up*	*Type*	*Time (mo)*	*No.*	*Survival (%)*	*Time (mo)*	*No.*	*Survival (%)*	*Time (mo)*	*No.*	*Survival (%)*
Behrendt et al. *(77)*	Dutch ALL II-V	5-yr survival	CNS	<24	103	—[a]	25–36	19	—[a]	>36	4	—[a]
Gaynon et al. *(76)*	CCG 100 (1985–1989)	6-yr EFS	CNS	<18	102	24	18–35	84	44	>36	34	59
			Combined	<18	34	9	18–35	26	11	>36	60	48
Schroeder et al. *(116)*	Scandinavia (1981–1986)	1994	CNS	<24	32	21	24–36	21	38	>36	16	61
Wheeler et al. *(75)*	UKALL X (1985–1990)	5 yr EFS	CNS	<24	62	24	24–30	23	35	>30	13	34
			Combined	<24	18	11	24–30	13	15	>30	23	41

Abbreviations: EFS, event-free survival; CNS, central nervous system; CCG, Children's Cancer Group.
[a] Separate survival analyses not performed (overall survival estimate 25% at 5 yr, EFS 10–15% at 5 yr.

Table 3
Treatment for CNS relapse in ALL

Authors	*No. of patients*	*Treatment*	*Outcome*
Land et al. *(104)*[a]	36	TIT and craniospinal irradiation	No difference in CNS relapse rate between groups
	51	TIT and cranial irradiation	
Mandell et al. *(117)*	10	TIT and delayed craniospinal irradiation	7/10 patients in CR
Ribeiro et al. *(68)*	20	TIT and craniospinal irradiation	70% 5-yr EFS
Winick et al. *(105)*[b]	120	TIT and cranial irradiation	46% 4-yr EFS
Messina et al. *(118)*	41	Variable intensified chemotherapy and cranial irradiation	12.6% 5-yr EFS
	19	Total body irradiation and allogeneic BMT	56.3% 5-yr EFS
Ritchey et al. *(106)*	83	Intensive systemic and IT chemotherapy with delayed craniospinal irradiation	71% 4-yr EFS overall

Abbreviations: TIT, triple intrathecal chemotherapy; CR, complete remission; EFS, event-free survival; CNS, central nervous system; BMT, bone marrow transplantation.
[a] Randomized CNS treatment.
[b] Randomized systemic treatment.

ABMT could be an appropriate treatment for patients with an isolated extramedullary relapse *(112)*, and the small comparative study in Table 3 has been interpreted as comparing favorably with chemotherapy. Most transplant units have a similar small cohort of long-term survivors following ABMT after extramedullary relapse.

Because critical review of the available evidence *(113)* suggests that there is no established indication for ABMT in the management of relapsed ALL, the only justification for this therapy would seem to be in the context of a randomized trial. Most ABMT regimens for lymphoid leukemia incorporate total-body irradiation, which carries all the disadvantages of spinal irradiation and significant additional late effects of therapy. There would appear to be more justification for the use of allogeneic transplantation in selected cases of early relapse in the hope of reducing the chance of recurrence. It is the author's practice to consider BMT from a histocompatible sibling or a closely matched volunteer unrelated donor for children with an early CNS relapse (<24 mo) and other adverse prognostic factors, such as initial leukocyte count > 50×10^9/L, but there is no firm evidence to support this recommendation.

There is a dearth of consistent protocols for the management of CNS relapse and, in particular, of randomized trials. The combination of systemic intensification, IT chemotherapy, and cranial irradiation is a reasonable approach to treatment. Since most children relapsing will not have received previous cranial irradiation, the morbidity should be acceptable. BMT is probably justified for patients with early relapse.

3.8. Ensuring Adequate and Sensitive Long-Term Follow-Up

Clinical research is a long-term enterprise, and nowhere is this more apparent than in the study of late effects of therapy. Many of the important late effects of therapy for ALL have been related either to efforts to prevent CNS relapse or perhaps to the influence of subclinical leukemic infiltration in the CNS. Although good evidence implicates cranial irradiation in the genesis of brain tumors, precocious puberty, and growth hormone insufficiency, neuropsychological sequelae will not be eliminated merely by abolishing cranial irradiation.

Further studies are needed on the relative neurotoxicity of alternative CNS-directed therapies, in particular combinations of parenteral and IT MTX. Wider use of dexamethasone may increase the incidence of growth retardation and bony complications, such as avascular necrosis of the hip, whereas reliance on high-dose etoposide may increase the incidence of secondary leukemias, a complication developing in 2 of 83 children in a recent POG study of CNS relapse *(106)*.

Long-term follow-up studies, particularly those involving neuropsychological assessment, are expensive and time-consuming. Nevertheless, if CNS-directed therapy is to be optimized, all front-line studies should, as a minimum, provide guidelines for the evaluation of late effects. Further work is needed to ensure effective follow-up of long-term survivors without increasing their sense of vulnerability and dependence.

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12 Diagnosis, Prophylaxis, and Treatment of Central Nervous System Involvement in Acute Lymphoblastic Leukemia

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CONTENTS

1. INTRODUCTION

Following the first successful treatment of acute lymphoblastic leukemia (ALL) in children in the 1960s, it soon appeared that relapses occurred frequently in the cerebrospinal fluid (CSF), with 50–80% of patients affected in most series. This led to the designation of the CSF as a *sanctuary site*, where leukemic cells were sequestered behind the blood-brain barrier from the effects of chemotherapy *(1)*. Various approaches were tried to overcome this barrier. The first successes came with the use of craniospinal irradiation, and then with the combination of cranial irradiation and intrathecal methotrexate (IT MTX) administered by lumbar puncture. This method was the standard during the 1970s, but it decreased in favor as long-term side effects soon became apparent: intellectual impairment *(2,3)*, decline in growth rate *(4)*, and the occurrence of brain tumors as second malignancies *(5,6)*.

These side effects were especially noticeable in children irradiated at younger ages. The extended use of intrathecal medication (MTX alone or in combination with cytarabine and a corticosteroid) was found to be a good alternative to cranial irradiation *(7)*. A third way to overcome the blood-brain barrier was the use of high-dose MTX, infused over 6–36 h, with leucovorin rescue *(8)*. From the beginning, prednisone was the mainstay of remission induction treatment, but in the early 1970s a randomized study substituting dexamethasone for prednisone *(9,10)* showed that the alternative steroid can decrease the number of CNS relapses, even though the event-free survival (EFS) rates in this trial were similar.

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Risk factors for CNS relapse include a high leukocyte count at diagnosis, mature B-cell phenotype, T-cell phenotype, Philadelphia chromosome-positive ALL, and, of course, CNS leukemia at diagnosis *(11)*. Infants also have an increased incidence of CNS relapse, whereas the motility of leukemic blasts (hand-mirror cells) seems to confer a lower risk for such relapse, presumably because these cells can migrate back into the bloodstream and become susceptible to systemic therapy *(12)*. Because CNS relapse rates have decreased to 2% or lower in several recent studies *(13)*, the impact of almost all these prognostic factors has been greatly reduced.

2. CURRENT STATE OF THE ART

2.1. Clinical Signs and Symptoms

Most often initial CNS involvement in ALL is clinically silent. If signs and symptoms do occur, they are frequently related to increased intracranial pressure *(8)*. Otherwise, visual disturbances, blindness, myelopathy, cranial nerve palsy, and a hypothalamic syndrome may occur. Radicular pain can be a troublesome symptom. Cranial nerve palsies may exist with or without blasts in the CSF, and the same goes for intraocular manifestations, either in the retina or in the anterior chamber of the eye. The occurrence of convulsions should raise the suspicion of brain or meningeal involvement, but most often no specific cause is found. Treatment toxicity is generally held responsible for convulsions, but it can also be the result of CNS invasion.

2.2. Diagnosis of Initial CNS Involvement

Criteria for the diagnosis of CNS involvement vary from "any number of blasts in cytospin preparations" *(14)* to "more

than five mononuclear cells per microliter with unequivocal blasts in cytocentrifuge samples" *(15)*, which is extended by some to include: "on two successive occasions" *(16)*. Imaging techniques such as computed tomogrpahy or magnetic resonance imaging scanning have only limited value in the diagnosis of CNS involvement *(17)*. An exception would be the rare occurrence of lymphomatous brain lesions.

2.3. CNS-Directed Presymptomatic Therapy

The first successful presymptomatic treatment of meningeal involvement consisted of cranial irradiation together with IT MTX *(1)*, a strategy that was widely used during the 1970s and for most of the 1980s *(1)*. A Pediatric Oncology Group (POG) study demonstrated that extended triple IT medication with MTX, cytarabine, and prednisolone was as effective as radiotherapy and that 1 yr of IT treatment was as good as 3 yr *(7)*. The IT dosage regimen was later adapted to age rather than weight or body surface area, since age correlates better with the volume of the meningeal space *(18)*. Although, in some studies, cranial irradiation gave better results than intermediate dose MTX 3 × 500 mg *(19,20)*, the Children's Cancer Group (CCG) found that in high-risk patients, at 2–3 yr of follow-up, the EFS curve of the cranial irradiation group was superior, but the curves later crossed, and iv plus IT MTX gave better results after 4–6 yr of follow-up *(21)*. The superior results were not related to fewer CNS relapses but to fewer bone marrow relapses. Also, comparison of the Italian *(22,23)* and Dutch *(24)* variants of the Berlin–Frankfurt–Münster (BFM) protocol with the German BFM protocol indicated that cranial irradiation can effectively be replaced by iv and IT medications. Several groups have found that early intensification of IT chemotherapy, together with intensive systemic treatment, virtually eliminates CNS relapse in children with ALL *(25)*.

2.4. Interaction of Systemic and CNS-Directed Therapy

Systemic therapy is all important in preventing CNS relapse. In an early randomized study, the Cancer and Leukemia Group B (CALGB) group demonstrated the importance of the type of steroid used in systemic regimens: dexamethasone proved superior to prednisone in preventing CNS relapse *(9,10)*. In some studies, high-dose MTX seemed more protective against systemic than CNS relapse *(19,21)*. In the UKALL XI study, however, MTX at 6–8 g/m^2 in addition to 16 doses of IT MTX reduced the CNS relapse rate (from 5 to 2.5%), without significant improvement in the EFS rate at 4 yr *(26)*.

2.5. Treatment of CNS Relapse

EFS rates of 12–70% have been reported for patients who received various treatments after CNS relapse, almost all encompassing cranial irradiation *(27–31)*. The group with the best prognosis *(30)* included patients with CNS2 disease (i.e., <5 cells/mm^3 with identifiable blasts), which makes comparison with other groups difficult. Most of these series had few patients. The only larger group of children with CNS relapse was described by POG investigators. Of 120 patients treated with 2400 cGy cranial irradiation and triple IT therapy, 47% were event-free survivors at 4 yr *(27)*. The time to initial CNS relapse is the most important factor in determining subsequent prognosis *(32,33)*. A bone marrow relapse is the most frequent event after a first isolated CNS relapse *(33–35)*, providing a rationale for intensive systemic treatment of the latter *(36)*. The validity of this approach is underscored by the finding that, with sensitive techniques, minimal residual disease can be found in the bone marrow of almost all patients with CNS relapse *(37–39)*.

2.6. Side Effects and Late Effects

Neuropsychological late effects after leukemia treatment became obvious in the late 1970s and early 1980s *(2)*. At that time, it was concluded that children without initial CNS involvement generally do well later. Younger children, however, generally had a worse outcome than older children. Whether cranial irradiation was causing more late effects than other forms of presymptomatic CNS treatment was, and still is, a matter of some debate. Evaluation of this issue is complicated because the interaction among IT therapy, systemic therapy (especially iv MTX), and radiotherapy determines outcome *(3)*. Most recent reports find the highest incidence of neuropsychological damage in younger children after cranial radiation *(40–42)*. Girls seem to be more susceptible than boys *(42,43)*. Academic achievement and employment is negatively influenced by cranial irradiation *(44)*. A small prospective study of 23 children treated with 18 Gy of cranial irradiation and 26 receiving multiple doses of MTX (1 g/m^2) showed a small decline in test results in both groups but no significant difference between the groups *(45)*. The cumulative dose of MTX seems to be important in this respect. Generally, late effects are more pronounced in patients with initial or relapsed CNS leukemia, as these children have received more CNS-directed therapy than those receiving presymptomatic treatment only *(46)*.

Endocrine consequences of treatment have been reviewed by Shalet *(47)*. Final height is negatively influenced by cranial irradiation, and this effect is obviously more pronounced in young children, who have the most growing to do *(4,48)*. Obesity after treatment is more obvious in females, particularly those who did not receive cranial irradiation *(49)*. Surprisingly, obesity was more often associated with a lower dose (18 Gy) of cranial radiation *(50)*. Menarche tends to come earlier in girls irradiated before age 8 years, and a reduced menarche rate is associated with radiation doses exceeding 24 Gy *(51)*. Sexuality did not differ appreciably between patients and controls, but "more restrictive attitudes" were noted among patients *(52)*. Other late effects include a reduced bone mass, which was found to be related to cranial radiation *(53,54)*. More surprisingly, subclinical restrictive deficits in pulmonary function were found and were also more frequent in cranially irradiated younger children *(55)*. One of the most worrisome late effects is the development of secondary malignancies *(56)*. Brain tumors, for example, are related to cranial irradiation *(57)*, especially in younger children *(5)*, with a contribution from the interaction with systemic therapy, epipodophyllo-toxins in particular *(6)*.

3. CONTROVERSIES AND PROBLEMATIC ISSUES

3.1. Diagnosis of Initial Meningeal Involvement

Initial CNS involvement is present in 2–5% of the entire group of children with ALL. The reported incidence will vary depending on the diagnostic criteria used. It is low (1–2%) in

children with standard-risk ALL and higher (up to 14%) in high-risk cases *(58)*. Meningeal involvement is more likely to occur in patients with a T-cell or mature B-cell phenotype and in those with bulky disease *(8)*.

The standard diagnosis of meningeal involvement, as defined at the Rome workshop, requires both an elevated cell count (>5/mm^3) and unequivocal blasts on a well-stained cytospin preparation *(15)*. In the St. Jude protocols, a low number of blast cells in the CSF was associated with a significantly higher CNS relapse rate; at 5 yr the CNS relapse-free survival estimate was 87% vs 96% for patients without CSF blasts at diagnosis *(59,60)*. The CCG *(61)* and the Dutch Childhood Leukemia Study Group (DCLSG) *(62)* did not find a significant difference in outcome between patients with or without low numbers of blasts. These conflicting reports may reflect differences in therapy [CNS-directed (cranial irradiation or extended IT medication) as well as systemic]. About 10% of initial CSF specimens contain contaminating erythrocytes, making a definite diagnosis of CNS involvement impossible. Several authors report that patients with blood contamination in their initial CSF sample had a worse outcome *(63,64)*. Interpretation of these data is complex, because patients with erythrocyte-containing CSF samples tend to have more unfavorable prognostic characteristics *(64)*.

Apart from the cytologic examination, other tests have been described to ascertain the presence or absence of leukemic blasts in CSF. In cases complicated by viral illness, the discrimination between reactive and leukemic cells can be very difficult. Immunophenotyping may support the diagnosis *(65–67)*, as may DNA cytophotometry *(68)*, cytogenetics *(69)* and polymerase chain reaction (PCR) methods *(70)*. Other methods, including the determination of β-2-microglobulin *(71)* and other soluble markers, probably lack the specificity to be clinically useful. For instance, although β-2-microglobulin is increased in patients with CNS leukemia, it is also increased after cranial irradiation, following intrathecal therapy, and in cases with viral infection *(72)*.

The diagnosis of meningeal involvement in a newly diagnosed patient with ALL may be difficult, especially if there are circulating blasts and the CSF specimen shows contamination with erythrocytes. In some patients, this finding results from a traumatic lumbar puncture, but it is not unusual to see a perfect lumbar puncture deliver a sample contaminated with erythrocytes (and blasts). It is quite possible that the erythrocytes were present in the CSF as a consequence of meningeal involvement. Seemingly trivial circumstances may also influence the erythrocyte count in CSF: with a minimally traumatic puncture, the first few drops of CSF may be tinged, with the next milliliters showing progressive clearance. Often, a repeat CSF specimen can only be obtained after therapy has started, because steroids given systemically, with or without IT therapy, may have cleared the CSF. This author therefore advocates delaying IT therapy until the first to third day after the initial diagnostic spinal tap. Even when blood contamination in the first CSF sample confers a higher risk of relapse, it has still to be demonstrated whether this hazard can be avoided by giving IT therapy once with the very first lumbar puncture. The initial lumbar puncture, especially when it is used for IT medication, is an important one and should be performed by a experienced clinician, preferably with the use of general anesthesia.

3.2. Diagnosis of CNS Relapse

CNS relapse is more frequent in certain biologic subgroups of patients, but treatment remains the single most important prognostic factor *(14)*. The incidence of this complication depends on the diagnostic criteria used. With the ominous implications of a CNS relapse, clinical management decisions should be based on firm grounds: no harm is done by serial lumbar punctures performed without alteration of therapy, as time invariably confirms the true positives *(16)*. How often should one perform a spinal tap, and for how long after diagnosis? With prolonged IT medication, CSF samples will automatically become available, often once every 4–8 wk. The yield of surveillance CSF examinations will be very low, however. Among >12,000 samples, only 0.8% provided a diagnosis of meningeal relapse *(61)*. The number of CNS relapses later than 3 yr after diagnosis will be even lower with current treatment protocols. On the other hand, the prognosis of a CNS relapse is decidedly worse when higher CSF cell counts (>100/mm^3) are present *(33)*. Also, meningeal relapses that are detected once clinical symptoms have appeared probably have a worse outcome. Surveillance lumbar punctures seem reasonable during therapy, and maybe even until 1 yr after stopping treatment. Routine lumbar punctures later than 3 yr after diagnosis (or 1 yr after cessation of chemotherapy) do not seem warranted, because at this time the number of false positives can become higher than the true positives. For surveillance, the determination of CSF cell counts appears adequate, with other methods of detecting leukemic cells reserved for a repeat spinal tap. Given the serious consequences of relapse treatment, a prudent clinical decision would be not to rely on a single follow-up CSF sample, but to repeat the spinal tap after 2–3 wk. If a viral infection is responsible for the increased cell count with suspect cells in the CSF, additional time will be needed to allow the infection to abate. On a second spinal tap, diagnostic tests other than cell count and cytospin should be used. Immunophenotyping and PCR techniques to detect minimal residual disease can be very useful in confirming or refuting a diagnosis of meningeal relapse. Other CSF determinations (β-2-microglobulin or ferritin) should never be used alone to establish a diagnosis of meningeal relapse.

3.3. Presymptomatic CNS-Directed Therapy

Most children with ALL seem to be adequately protected from CNS relapse without cranial irradiation *(7,8,18,61,73–75)*. Whether this is also true for high-risk patients, including those with the T-cell phenotype, remains in question. The experience of the Italian *(22,23)* and Dutch *(24)* groups with a BFM-based protocol indicates that even in high-risk cases (including T-cell ALL), treatment without cranial irradiation can give comparable results to a similar protocol with cranial irradiation. Also, the Japanese Children's Cancer and Leukemia Group found equivalent results for IT medication and 18 Gy of cranial irradiation *(76)*. In recent St. Jude protocols, early intensification of IT therapy almost eliminated CNS relapses *(13)*. Since the results obtained with cranial irradiation and IT medication appear to becomparable in terms of

EFS and since in most reported series cranially irradiated children had worse neuropsychological deficits than children given IT therapy, indications for the use of cranial irradiation, especially in younger children, appear very limited. With present-day protocols, even high-risk groups are not likely to benefit from cranial irradiation, which should be reserved for the few patients with meningeal relapse. Postponement of irradiation will also limit the late effects in this high-risk group.

3.4. Role of Systemic Therapy in Preventing CNS Involvement

A randomized study of the CALG B group in the early 1970s demonstrated fewer CNS relapses in dexamethasone-treated patients than in those given prednisone, although overall EFS did not differ significantly *(9,10)*. Better CNS protection was also found in a nonrandomized Dutch ALL-VI study (1984–1988) of non-high-risk patients and historical controls, in which the overall EFS was improved to 81% *(77)*. The superiority of dexamethasone was conclusively established in a recent large randomized study of the CCG *(78)*. The difference in effectiveness between these closely related steroids may have two explanations. First, dexamethasone penetrates the blood-brain barrier more readily than prednisone *(79)*, and although it is usually given in a sevenfold lower milligram dose than prednisone, its in vitro cytotoxic effect was found to be stronger than that *(80,81)*. The side effects of dexamethasone seem to be more pronounced than those of prednisone, especially the Cushing effect and mood changes during therapy. The fear of avascular necrosis of bone, however, was not borne out in the Dutch ALL-VI study: none of 166 children with non-high-risk ALL who received a high cumulative dose of dexamethasone had this side effect, compared with 9 of the 374 treated on a BFM-like protocol *(82)*. Avascular necrosis of bone seems to be more correlated with intensive treatment [either in high-risk cases or in those treated for relapse *(83)*] and with age >12 yr *(82,84)*.

Does high-dose MTX have a function in CNS prophylaxis? The answer may not be straightforward, as interactions among treatment modalities, both intrathecal and systemic, can strongly influence clinical outcome. Early trials with intermediate-dose MTX (500 mg/m^2 × 3) showed inferior CNS prophylaxis for MTX compared with cranial irradiation but better systemic control of ALL *(19,20)*. Other studies with high-dose MTX have failed to demonstrate superiority over cranial irradiation in the prevention of CNS relapses *(85–88)*. A recent randomized study indicated that high-dose MTX (2–4.5 mg/m^2) was less protective than cranial irradiation against CNS relapse, but the overall results were comparable *(76)*. Intermediate-dose (1 g × 17) iv MTX was less effective than triple IT therapy in preventing CNS relapse *(75)*. The duration of follow-up may be a crucial factor in such studies, as shown by the CCG experience, in which CNS relapse-free survival curves crossed after 4 yr of follow-up, and the EFS at 6 yr was better in the IT plus low-dose iv MTX group than in the cranial irradiation group *(21)*.

It is quite possible that the peak serum levels of MTX are less important than the exposure time. In a BFM study, 12 g given over 4 h, with rescue at 24 h, gave inferior results to 1 g given over 36 h with rescue at 48 h *(89,90)*. In an in vitro assay, leukemic T-ALL cells were resistant to a short (4-h) incubation with MTX but were as sensitive as leukemic B-cell precursors to sustained (24-h) incubation with the drug *(91)*. In a recent French study, high-dose MTX did not prove superior to low-dose MTX, in either standard-risk or high-risk patients *(92)*. High-dose MTX may still be of benefit in high-risk patients, especially if they have the T-cell or mature B-cell leukemic phenotype.

Reinduction treatment seems to be an important component of strategies to prevent CNS relapse, even in standard-risk patients. With omission of reinduction protocol II in a BFM-like study, 11% of children had a CNS relapse, with this proportion dropping to zero after introduction of protocol II *(24)*, which includes dexamethasone as a steroid, in contrast to the use of prednisone during the induction phase. Addition of a second dexamethasone-containing reinduction course decreased the incidence of CNS relapse from 5.1% to zero *(21)*. However, addition of high-dose cytarabine to the post-induction regimen did not significantly reduce the incidence of CNS relapse *(93)*.

Systemic therapy is all important for the prevention of CNS relapse. Dexamethasone is now used up-front in many protocols or as a reinduction agent, and studies comparing the efficiency of dexamethasone vs prednisolone are ongoing. The tradeoff in toxicity will be an important consideration in evaluating these agents, but use of a more potent steroid could allow dose reductions in other potentially damaging drugs. Other steroids have not been systematically tested in ALL.

The use of high-dose MTX does not seem to have had a large impact on the occurrence of CNS relapse. It is theoretically possible that IT MTX, acting through slow release from the CSF, could produce considerable systemic effects, partly explaining the good results of several CCG and French studies that did not use high-dose MTX.

3.5. Treatment of Initial Meningeal Involvement

In most current protocols, unequivocal initial meningeal involvement is treated as high-risk disease with use of cranial irradiation. The necessity for this approach is questionable, and evidence-based recommendations are not available. Hence, there is a need to assess the value of cranial irradiation in children with initial meningeal involvement. For instance, patients who clear their CSF of blasts within the first 4 wk of induction treatment may not need cranial irradiation, an issue that is being addressed in the current Dutch ALL-9 protocol. Several (young) patients with initial CNS involvement in the author's clinic, who were treated on a Dutch ALL-VI-based (amended) protocol, were not irradiated because their parents declined that form of treatment. They are doing well without relapse 10 or more years later.

There is insufficient information on the treatment of initial meningeal involvement to answer several outstanding guestions. Is cranial irradiation necessary when dexamethasone is used as the steroid? Should patients with normocellular CSF and identifiable blasts or those with contaminating erythrocytes and blasts be treated as having meningeal involvement? At present, most protocols do not provide for any special treatment of such patients.

3.6. Treatment of CNS Relapse

Isolated meningeal relapse requires vigorous systemic retreatment, and in almost all protocols local control is attempted by cranial irradiation. However, some children will have had cranial irradiation before, posing definite management problems. Even so, it may be possible to avoid cranial irradiation in these cases. The use of an intraventricular access device (Ommaya reservoir) to deliver IT medication has yielded better results than lumbar administration in some clinics *(94,95)*. Such devices have sometimes been used in conjunction with limited-dose (6–9 Gy) neuraxis radiation *(96)*. Good results were also obtained with a Rickham reservoir (posteriorly located) in 10 children with multiply relapsed CNS leukemia: 9 of the10 achieved complete remission *(97)*, with 7 remaining in remission after a follow-up of 77–153 mo (K. Hählen, personal communication). In other comparisons of intraventricular and lumbar administration, 8 of 9 adult patients treated with an Ommaya reservoir became free of blasts vs 4 of 12 treated with lumbar administration, but these results were not significantly different *(98)*.

Few solid conclusions can be drawn from these studies, as the numbers of patients are low and a selection bias may have been present. Also, the neuropsychological status of these children is not fully reported, preventing a comparison with the late effects in irradiated children.

Another issue is whether or not to irradiate the spinal meningeal space in children with CNS relapse. An important consideration in the decision should always be the age of the patient. The younger the child, the more a treatment without radiation would have to be considered. A therapy with an acceptable risk-benefit ratio for a 16-yr-old patient may not be acceptable for a 3-yr-old.

The place of bone marrow transplantation is presently not clear. The BFM group found no significant differences in EFS rates between patients receiving transplants and those treated with chemotherapy *(99)*. In an Italian study, autologous bone marrow transplantation gave better results in 19 patients than in 41 historical controls, but the latter group had a very poor prognosis (19% EFS) *(31)*. Intermittent cranial radiotherapy, which has been used in a small number of patients with CNS relapse ($n = 9$) appeared to be well tolerated and led to long remissions in some cases *(100)*.

In a few cases, medications other than the classical trio (MTX, cytarabine, and steroids) have been used. Intrathecal injections of 6-mercaptopurine are feasible in a dose of 10 mg twice weekly and produced remissions in four of nine multiply relapsed adult patients *(101)*. Also, etoposide IT proved more effective than high-dose etoposide iv in two patients *(102)*. Intrathecal administration of radionucleides has never become popular, although it seems a sensible way of providing radiotherapy to the meninges but not to the brain. Successes have been reported with radioactive gold (^{198}Au-colloid) *(103)* and antibodies labeled with 131-iodine have demonstrated activity in the treatment of meningeal neoplasia *(104)*.

With a first CNS relapse in a previously nonirradiated patient, standard care would include cranial irradiation, but not spinal irradiation. Intraventricular use of chemotherapy is an interesting option, but it needs more supportive evidence and follow-up study. In view of the reasonably good results obtained with this strategy, especially in patients with late CNS relapses, bone marrow transplantation would not be considered an appropriate alternative treatment.

Clinical decision making has to balance the chance of cure with the probability of adverse late effects. Such decisions need to be supported by evidence obtained in protocol-controlled studies of large series of patients. The lack of compelling evidence in many areas can be traced partly to differences among definitions of risk groups and diagnostic criteria, as well as the failure to report not only EFS but also the quality of cures. Given the low numbers of patients who now experience CNS relapse, only large-scale cooperation among study groups would be expected to provide answers to the questions raised earlier.

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CHEMOTHERAPEUTIC STRATEGIES

II

F

RELAPSED ACUTE LYMPHOBLASTIC LEUKEMIA

13 Treatment of Relapsed Acute Lymphoblastic Leukemia

Perspective 1

HELMUT GADNER, GEORG MANN, AND CHRISTINA PETERS

CONTENTS

1. EPIDEMIOLOGY AND PATHOGENESIS

With a constant rate of about 25–30% after adequate first-line chemotherapy, relapsed acute lymphoblastic leukemia (ALL) as an entity accounts for 9.3 cases per million children each year, representing one of the most common pediatric malignancies *(1)*. So far, few epidemiologic data generated from relapse therapy studies have been available. These are difficult to interpret because of a lack of randomized trials, selection biases, and small numbers of patients. Various factors, therefore, may account for essential differences between patient cohorts at relapse and initial diagnosis *(2,3)*. Patients with poor risk features at diagnosis are overrepresented, as expected, and the higher median age at the time of relapse reflects a higher relapse probability in older patients. However, most relapses occur in patients who lack well-established unfavorable diagnostic markers at first diagnosis.

The bone marrow is the most common site of relapse, followed by the central nervous system (CNS) and testes; in rare cases relapse occurs in other sites, such as the eye, ovary, or skin. Relapse may evolve as an isolated event or in combination with other sites (e.g., bone marrow and CNS or testes). Recently published reports have shown an isolated bone marrow relapse incidence in the range of ≤20%, isolated recurrences in CNS and testes between 6 and 2%, and combined relapses in <4% of patients *(4–13)*. All forms of recurrence have to be considered as variants of a systemic disease *(14–16)*. Different mechanisms may favor relapse in childhood ALL *(17)*. For example, (1) anatomic barriers may impede the penetration of antileukemic drugs in therapeutic concentrations into "sanctuary sites," such as the CNS and testes; (2) leukemic cells, either by intrinsic properties or by altered enviromental conditions, may remain in the G0 phase and may therefore not respond to chemotherapy; and (3) primary or secondary resistance to antileukemic therapy may prevent cells from undergoing apoptosis *(18,19)*. As a consequence, leukemic blast cells may persist, allowing the emergence of resistance and proliferation *(16,20)*. The pattern of systemic relapse following an extramedullary recurrence strongly suggests that such recurrences may be caused by reseeding of leukemic cells from the CNS or testes into bone marrow *(21,22)*.

2. DIAGNOSTIC CONSIDERATIONS

The symptoms and signs of a child with ALL in relapse are usually less prominent than those seen at initial diagnosis, owing to early detection by periodic surveillance, but they often reflect the original disease pattern. Careful physical examination and documentation of relapse sites are therefore mandatory. This includes bone marrow aspiration (or trephine

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biopsy), lumbar puncture, and imaging of the neuroaxis, testes, and eyes, as well as the lymphatic organs (lymph nodes, thymus, liver, and spleen) and kidneys.

2.1. Definitions

Isolated bone marrow relapse is diagnosed if ≥25% unequivocal blasts are found in bone marrow (M3 marrow), without extramedullary involvement. Marrow involvement with proven leukemia at extramedullary sites and 5% or more leukemic cells in bone marrow denote a combined bone marrow relapse. An isolated extramedullary relapse represents disease manifestation outside of the bone marrow, with bone marrow containing <5% blast cells (M1 marrow) *(17,21)*. These definitions are based on cytomorphology and may be challenged as soon as more sensitive immunologic, cytogenetic, and molecular methods become commonly available for the detection of malignant cells below the morphologic level *(23,24)*.

Usually meningeal recurrence (CNS3 status) is evidenced by detection of >5 blast cells/μL in the craniospinal fluid (CSF). Immunologic or cellular/molecular genetic studies may be helpful in distinguishing leukemic cells from reactive lymphocytes *(23,25)*. The significance of ≤5 cells/μL of CSF detected by cytospin (CNS2) remains controversial *(26–28)*. Testicular relapse usually presents with unilateral or bilateral enlargement of the testes (*overt relapse*). Cytomorphologic or histopathologic confirmation is recommended for relapse documentation. Elective testicular biopsy is no longer advocated for occult disease, since it has not shown any benefit for the treatment of patients with occult relapse during therapy or off therapy *(29–33)*.

2.2. Morphology and Immunology

The morphologic and immunologic features of ALL at relapse are usually not different from those at initial diagnosis. In one-third of patients, however, a shift from the small French–American–British (FAB) L1 blast cells to the larger FAB L2 type has been observed. Similar observations have been made for cytochemical changes (i.e., positive or negative shift in the periodic acid-Schiff score, detectable in approx 30% of children at relapse), appearance of positive cytoplasmatic stains for myeloperoxidase, and others *(34–36)*. Several investigators have also demonstrated changes in the expression of cell markers (immunologic shift) in the range of 25% to >50% of patients showing an intralineage or interlineage shift *(34,36–38)*. This phenomenon may emerge from progenitor cells capable of differentiating into more than one lineage (lineage promiscuity) or from inappropriate or aberrant gene activation (lineage infidelity) *(23,39,40)*. The most commonly detected shifts are toward a more undifferentiated immunophenotype, with loss of HLA-DR or CD10 antigen *(34,36)*. In many cases these leukemias can be classified as mixed lineage, biphenotypic, or acute hybrid leukemias *(41)*. In some cases the distinction between ALL and acute myeloid leukemia or between relapse and secondary leukemia may become difficult *(42)*.

2.3. Cytogenetics and Molecular Biology

The detection of chromosomal abnormalities by newer methods of genetic analysis, including fluorescence *in situ* hybridization (FISH) techniques, is possible in >90% of ALL cases in relapse *(43)*. Only a few studies, based on small numbers of patients, have systematically compared cytogenetic changes at diagnosis with those at relapse *(34,37)*. This spectrum reflects the variety of alterations in chromosome number and structure seen at primary diagnosis, although in approxi 30% of cases changes of karyotype are detected. These are usually minor and range from normal to pseudodiploid or hyperdiploid, or *vice versa (34)*. Most of the described changes derive from clonal evolution or clonal selection of an independent drug-resistant cell, which eventually develop into a clonal proliferation responsible for relapse *(20,39)*. This explanation is most applicable to cases with major changes, in which replacement of the original clone with a new transformed karyotype (e.g., chromosomal translocation involving 11q23 or 12;21) was observed and led to a secondary leukemia *(37,40)*.

By using molecular biologic and genetic techniques in relapsed disease, it is possible to detect important genetic rearrangements that cannot be detected by routine karyotyping (e.g., *TEL-AML1*) or to find rearrangements involving different genes in the same genetic region (e.g., the *MLL* gene) that translate into biologically diverse entities *(23,43–45)*. Not surprisingly, genetic features associated with a poor prognosis are found comparatively more often in relapse. For example, the translocation t(9;22), or Philadelphia chromosome (Ph), generates the chimeric *BCR-ABL* oncogene, the second most common fusion event in relapsed ALL, ocurring at a frequency (12%) about three times higher than in newly diagnosed patients *(46)*. Complex chromosomal abnormalities with duplication of the long arm of chromosome 1 as an additional abnormality are also found with relatively higher frequency in relapsed patients *(47,48)*.

One exception is the fusion transcript *TEL-AML1*, representing the most frequent gene rearrangement arising from a cryptic translocation, t(12;21), in patients with relapsed childhood B-lineage ALL. The frequency of this abnormality, 24%, is similar to that seen in newly diagnosed patients *(49,50)*.

2.4 Risk Factors

Several factors influence the ability to achieve second remission and prolonged disease-free survival (DFS) after ALL relapse *(15,17)*. These have emerged from long-term observation of multicenter ALL relapse trials; despite some overlap, they are not identical to those associated with the prognosis at initial presentation *(21,50–55)*. The identification of these factors is essential for treatment stratification in clinical trials.

2.4.1. Time to Relapse

Patients who relapse while on chemotherapy have less chance of attaining a second remission and have shorter remissions than do patients with recurrences after cessation of therapy. The likelihood of successful remission induction is also greater in patients with longer preceding remissions *(56)*. Finally, DFS in patients who relapse off therapy is proportional to the length of remission before relapse occurred *(57)*. Sadowitz, et al *(55)* reported a superior outcome in patients with relapse occurring >6 mo after cessation of therapy, compared with that for patients relapsing earlier.

Recently, the results from an unselected group of 774 heterogeneously treated relapse patients, previously enrolled in Children's Cancer Group (CCG) studies, have confirmed that late relapses (≥ 36 mo from diagnosis) are associated with better second event-free survival (EFS) and survival rates at 6 yr than are earlier relapses at any site. In contrast, earlier relapses (<18 mo and 18–35 mo from diagnosis) conferred a worse survival probability *(1)*. The Berlin–Frankfurt–Münster (BFM) ALL-Relapse Study Group, after analyzing 1188 patients previously treated in BFM or COALL studies and entered in three consecutive ALL relapse trials (ALL-REZ BFM 83, 85, 87), reported a 10-yr EFS probability (*p*EFS) of 38% in patients with late bone marrow relapses (≥ 30 mo from diagnosis), compared with 17% in those with early (within 18 to <30 mo) and 10% in those with very early relapses (<18 mo from diagnosis) *(21,53,58)*. These data have been confirmed recently by the UK experience (UKALLR1 study) *(59)*.

2.4.2. Site of Relapse

Besides the duration of first remission, the site of relapse has been referred to as the most important factor influencing prognosis. This has been clearly shown after retrospective evaluation of population-based patient cohorts or follow-up observations of patients relapsing after treatment in large frontline trials *(1,15,21,60)*. These reports confirm the widespread clinical impression that bone marrow relapse is the most important form of treatment failure in ALL *(1,21)* and signifies a worse prognosis (10-yr pEFS, 15% for isolated bone marrow relapse; 34% for combined marrow relapse) than does extramedullary involvement (44%) *(58)*. It has also been demonstrated that children with combined bone marrow relapse have a better prognosis than do those with bone marrow disease only if treated only by chemotherapy (7-yr pEFS, 42% vs 15% for combined vs isolated relapses) *(1)*. When late and early combined relapses were analyzed separately, EFS estimates in the BFM studies were superior to the results in combined relapse in both groups *(53)*. This contrasts with the assumption that combined bone marrow relapses translate into a better prognosis because they tend to occur later *(1)*. Isolated CNS relapses manifest more often within 3 yr from diagnosis, whereas testicular relapses usually occur when patients are off therapy *(1,15)*.

Moreover, for these extramedullary relapses, the prognosis is better when the relapse occurs after cessation of all chemotherapy rather than during therapy (6-yr pEFS, 72% vs 33% for late vs early isolated CNS relapse; 81% vs 52% for late vs early testicular relapse *[1,21,53]*). Extramedullary relapse has a superior outcome when not accompanied by a simultaneous hematologic relapse *(1,15,21,53,61)*.

These findings are somewhat surprising, as it is well understood that in children with isolated extramedullary relapse, molecular evidence of the disease is frequently found in the bone marrow *(16,22)*. As an example, the BFM ALL relapse studies have shown an EFS advantage for isolated extramedullary relapses treated with chemo/radiotherapy vs those with combined bone marrow relapses (44% vs. 34%) *(21;53;54;61)*. These data were also confirmed by a recent retrospective survival analysis among 1144 unselected, heterogeneously treated patients, who had been enrolled on CCG trials between 1983 and 1989 (6-yr survival rate, 49% for isolated CNS relapses, 70% for isolated testicular relapses, and 48% for combined bone marrow relapses) *(1)*.

2.4.3. Morphology and Immunology

Few data are available regarding the prognostic influence of morphology and/or immunophenotype observed at relapse. A morphologic or cytochemical shift, detectable in approx 30% of children at relapse, has not shown any significant impact on the duration of second EFS *(34,35)*. Univariate and multivariate analyses have not detected any changes in the prognosis at relapse in B-cell precursor ALL. For the T- cell immunophenotype, however, a strong prognostic value related to the outcome of retrieval therapy was found *(21,34,37,43,60)*. In some of these studies, T-cell immunology was associated with a poor prognosis only when the analysis was adjusted both for time and site of relapse *(1,38)*. In the BFM experience (ALL-REZ BFM 85), relapse of T-ALL at any site and at any time point was related to a dismal prognosis (*p*EFS after 5 ys, 9% vs. 26% in non-T-ALL), and no patient with T-ALL and relapse within 18 mo from diagnosis has survived beyond 15 mo *(21)*.

2.4.4. Cytogenetics and Molecular Biology

Commonly detected changes in karyotype at relapse [except the evolution to secondary leukemia *(37,62)*] are not necessarily associated with a worse prognosis. Usually, patients retain the same abnormalities that were seen at first presentation or remain cytogenetically normal *(34,47)*. Whether or not additional chromosomal abnormalities confer a worse prognosis at relapse than was assigned at presentation remains controversial *(63)*. In contrast, translocation t(12;21) or the corresponding *TEL-AML1* fusion transcript, which is found in about 20–30% of children with ALL at primary diagnosis, is associated with a favorable outcome after relapse (pEFS, 79% vs. 33% in *TEL-AML1*-negative patients) *(43,49,50)*. Recently, it has been argued that the strong association of the *TEL-AML1* chimeric product with hyperdiploidy >69 chromosomes might explain the prognostic advantage conferred by their translocation *(64)*. Certain other chromosomal translocations at relapse, such as the t(9;22) and t(4;11), are strong and independent predictors of induction failure and early recurrence *(46,51,65–68)*. Treatment responsiveness after first relapse leading to second remission is reported in only 60% of patients with Ph-positive ALL or *BCR-ABL* gene rearrangement, compared with 91% in those without *BCR-ABL* expression. Durable second remissions (median pEFS, 2 yr) were in turn found only in 2% vs 50% of these two subgroups *(46,51)*. t(4;11) or the presence of MLL gene rearrangements, which are frequent in infants, remains associated with a dismal prognosis. However, age also has an important prognostic impact, with significantly better EFS and overall survival rates in children 2–9 yr old than among infants and younger children, as well as patients 10 yr of age or older *(67)*. It has recently been shown that older patients with MLL rearrangement can expect longer second remissions is still unanswered (C.-H. Pui, personal communication).

2.4.5. Other Risk Factors

In addition to the duration of first remission, site of recurrence, immunophenotype, and cytogenetics, other features have

been associated with the likelihood of achieving a prolonged second remission in ALL. A peripheral blast cell count <10,000/μL or a white blood cell (WBC) count < 20,000/μL, as well as age >2 but < 10 yr at the time of relapse may confer a favorable prognosis *(17,54–56,69,70)*. In contrast, a WBC count ≥ 50,000/μL, age of ≥ 10 yr, hemoglobin ≥ 10 g/dL, and male sex might be associated with lower second EFS rate, when features are adjusted both for time to relapse and site of relapse *(1,55,60,69,71)*. Therapy is also an important risk factor in relapsed ALL. It has been shown that intensification of therapy can eliminate or alter the prognostic significance of some of the adverse prognostic features *(72,73)*. On the other hand, the intensity of previous treatment has not demonstrated a significant influence on survival following retrieval *(1,60)*.

3. CHEMO/RADIOTHERAPY AND RESULTS

3.1. Bone Marrow Relapse

3.1.1. Reinduction and Postinduction Chemotherapy

Relapse treatment has to rely on antileukemic drugs that have already been delivered during initial chemotherapy. The therapy usually includes aggressive multidrug reinduction chemotherapy followed by intensive consolidation and maintenance therapy. To prevent CNS relapse, a second course of CNS-directed therapy is needed. In former studies, standard three-drug induction with prednisolone, vincristine, and L-asparaginase has shown less effectiveness in inducing remission after relapse than at initial presentation *(74)*. Several current studies therefore implemented an anthracycline in the relapse approach, achieving a remission rate > 80% *(21,69, 71,75)*, but this practice was restricted by cardiotoxicity developing in the previously exposed patients *(76)*. In order to evaluate prospectively the impact of dose and drug of anthracycline on response, the CCG conducted a randomized trial proving that a weekly dose of idarubicin at 12.5 mg/m^2 is more myelotoxic than 45 mg/m^2 daunorubicin but is superior to daunorubicin and a lower dose of idarubicin (10 mg/m^2) with respect to the 2-yr EFS rate (27% vs 10% vs 6%) *(77)*.

The BFM Group used more intensive induction protocols including most, if not all, effective antileukemic drugs, thus avoiding the cumulative toxicity of anthracyclines. In four consecutive studies beginning in 1983, up to nine intermittent blocks of polychemotherapy [including, apart from the conventional drugs, high-dose methotrexate (MTX), high-dose cytarabine, ifosfamide, and etoposide] were given in combination with CNS-directed therapy [triple intrathecal (IT) therapy], followed by 24 mo of maintenance treatment *(21,53,54)*. With this induction regimen, a second remission was achieved in 96% of relapsed patients with a low or moderate peripheral blast cell count (PBC), whereas the remission rate was only 82% in children with a high PBC (>10,000 cells/μL) *(54)*. A similar high remission rate (97%) was reported by St. Jude Childrens' Hospital with use of an intensified rotational combination chemotherapy for patients in late hematologic relapse. Treatment consisted of an intensive five-drug reinduction therapy (6 wk) with CNS prevention, followed by continuation therapy with four pairs of drugs rotated weekly in 4-wk cycles over 120 wk *(70)*. Australian investigators, using an intensive chemotherapy protocol (Memorial Sloan-Kettering-New York II), reported a remission induction rate of 92.6% *(78)*. Less effective, however, was a combination of ifosfamide and etoposide, with an induction rate of only 40% *(79)*. Recently a Pediatric Oncology Group (POG) study demonstrated superior induction remission rates in childhood relapsed ALL comparing weekly PEG-asparaginase [97% complete remission (CR)] with biweekly dosing (82% CR) in a combination treatment with doxorubicin, prednisone, and vincristine *(80)*.

All the patients included in the four BFM ALL-REZ studies *(21,53,58)* could be divided into four risk groups, defined by their different chances of cure: (1) patients with late (>6 mo off therapy) extramedullary relapse had a pEFS of 77%; (2) those with very early (<18 mo after initial diagnosis) and early (within 18 and 30 mo after diagnosis) extramedullary relapses, combined (early and late) non-T-ALL relapse, and late non-T-ALL bone marrow relapses a *p*EFS of 35%; (3) those with early isolated non-T-ALL bone marrow relapse, a *p*EFS of 4%; and (4) patients with very early combined and isolated bone marrow relapse, and patients with T-ALL bone marrow relapse, had a median duration of remission of 3 mo only.

Similar dismal results were recently reported by the POG, evaluating the outcome of 258 patients in second remission. In the long-term follow-up, children with relapse on therapy or shortly after its cessation did not benefit from the intensified induction therapy; thus, only 10% of the patients remained continuously free of leukemia for 7 yr or more *(81)*.

3.1.2. CNS-Directed Preventive Therapy

As in newly diagnosed ALL, CNS-directed therapy is needed after recurrences to avoid further relapses *(15,82,83)*. Therefore, all protocols have incorporated intensive IT chemotherapy consisting of regular injections of MTX alone or in combination with hydrocortisone and cytarabine *(55,71,83,84)*. So far, the advantage of triple IT therapy has not been clearly proved *(85)*. Most investigators continue to rely on additional cranial radiotherapy. Although effective, this approach with doses of ≥ 24 Gy has been associated with neurologic sequelae and secondary malignancies, especially in preirradiated and young children *(86,87)*. Attempts have been made recently to reduce the dose of preventive cranial irradiation to ≤18 Gy (12 Gy in preirradiated patients) *(21,83)*.

Another important approach to prevention of CNS relapse in a patient with recurrent ALL was the intensification of systemic chemotherapy. In a POG study, no preventive cranial irradiation was given to children with late isolated bone marrow relapse (>6 mo after cessation of primary therapy) treated with a randomized, alternating non-cross-resistant combination chemotherapy. Only two CNS relapses among 109 patients were observed, whereas no treatment regimen (doxorubicin/prednisone vs cytarabine/tenoposide) was superior in terms of remission induction (97%) and 4-yr EFS rate (37%) *(55,71)*.

The BFM Group has also addressed, in a randomized fashion, the impact of high-dose cytarabine together with moderate or high-dose systemic MTX in preventing further CNS disease *(21,53)*. High-dose MTX (12 g/m^2 given as a 4-h infusion) failed to show an advantage over intermediate-dose MTX (1 g/m^2 as a 36-h infusion), and the randomization was stopped prematurely. Subsequently, the group has empirically

introduced additional cranial irradiation and triple IT injections for all children with bone marrow relapse after they observed a high rate of secondary CNS relapses following primary isolated bone marrow relapse *(83)*.

3.2. Extramedullary Relapse

3.2.1. Central Nervous System Relapse

The use of intensive polychemotherapy together with extended IT chemotherapy (single or triple) followed by dose-adjusted cranial or craniospinal irradiation (in accordance with the previously given radiotherapy) and maintenance IT therapy is considered the most successful therapy for CNS relapse *(14,88–91)*. Several factors make identification of the best treatment difficult. First of all, after adequate ALL front-line therapy with CNS-directed treatment, the overall incidence of isolated CNS relapse is low (between <5% and <10%), as reported in a recent survey of long-term results obtained from 12 large study groups worldwide *(13)*. Furthermore, the definition of CNS leukemia (>5 blast cells/μL in the spinal fluid, or few recognizable blast cells in the cytospin smear used for diagnosis of CNS relapse) has not been uniformly adopted in the various publications *(23,25–28)*.

Remission retrieval therapy for patients with isolated CNS relapse is largely influenced by whether or not the patient received cranial irradiation during initial therapy *(21,53)*. Fortunately, most patients included in the standard- or intermediate-risk group during front-line therapy do not receive irradiation and are therefore eligible for cranial or craniospinal radiotherapy at relapse *(6,10,12,89)*. Craniospinal irradiation (24 Gy) in combination with systemic chemotherapy can induce remission and a long duration of DFS (70% at 5 yr) *(91–93)*. It has been demonstrated, however, that effective CNS control can be achieved in most children without recourse to spinal irradiation, provided that intensified systemic therapy had been used *(55,71,90)*. To avoid prolonged myelosuppression and late effects of spinal irradiation, a different approach has been established by first inducing a CSF remission with triple IT chemotherapy, followed by irradiation of the cranial vault and the first three spinal bones at doses of 18 Gy *(21,53,84)* or 24 Gy *(55,71)*.

3.2.2. Testicular Relapse

The third most frequent site of extramedullary relapse in childhood ALL are the testes, usually presenting within a year after cessation of primary therapy. In previous studies with less intensive chemotherapy, the incidence in males was high (up to 50%, either isolated or concurrent with bone marrow involvement) *(17)*. With intensification of front-line treatment, especially since intermediate- or high-dose MTX has been introduced into treatment protocols, <5% of boys are victims of a testicular relapse *(69,94,95)*.

The standard therapy for late isolated or combined testicular relapse includes intensive systemic and intrathecal chemotherapy and local management *(52,96)*. For unilateral involvement, most investigators recommend bilateral irradiation or orchiectomy of the involved testis and irradiation of the contralateral testis (dose 24 Gy). In cases of bilateral involvement, irradiation of both testicles with 24 Gy or more may be indicated *(15,52,53)*. The duration of systemic treatment should be 18–24 mo, and intensification of therapy appears to be of benefit *(21,53)*.

As radiation therapy with 24 Gy and more is reported to be associated with deterioration of normal testicular function (sterility and impairment of endocrine function) *(17)*, recent attempts have been made to minimize these late effects by reducing the dose of radiotherapy to 18 Gy (BFM REZ studies) or even to omit testicular irradiation in favor of high-dose MTX with apparent continued testicular remission *(31)*.

3.2.3. Other Extramedullary Relapse

In contrast to the previously described relapse sites, relapse manifestations in other locations are rare and demand intensive polychemotherapy with local management and CNS-directed preventive measures, if feasible. Only a few recent relevant reports are available on this topic *(97,98)*.

Ovarian relapse usually presents with an abdominal mass and may occur within the first 3 yr *(98)*. Ocular recurrences, which often manifest together with local iritis, are frequently seen within the first year after cessation of therapy *(97,99)*.

There are no conclusive data in the literature on the value of local radiotherapy in rare extramedullary events in the ovary, bone, or soft tissue, for example. Generally, irradiation should be considered for residual or late responses.

4. ALLOGENEIC STEM CELL TRANSPLANTATION

Although allogeneic stem cell transplantation (SCT) is widely accepted as an appropriate therapy for children in relapse, several controversies exist as to whether or not a patient should be included in a bone marrow transplantation program *(100,101)*. In the absence of randomized trials, retrospective analyses performed by international registries [the International Bone Marrow Transplant Registry (IBMTR) and the European Group for Blood and Marrow Tansplantation (EBMT)] are used to answer some of the most important questions *(102,103)*. There is evidence that not only location of relapse, time to relapse, and other initial risk factors (immunophenotype, cytogenetic or molecular genetic abnormalities), but also pretransplant therapy, response to this treatment, and time to transplant are important factors in the selection of patients for this complex procedure *(104)*. The overall DFS after SCT from a matched sibling donor in second remission has been reported to range from 40 to 60% *(102,103,105–109)*. The most commonly used conditioning regimen consists of a combination of total-body irradiation (TBI; mostly applied in a fractionated manner) and cyclophosphamide or etoposide, or, less frequently, cytarabine *(108,110–112)*. Other regimens without TBI (e.g., myeloablative chemotherapy with cyclophosphamide and busulfan, preferably given to children younger than 2 yr of age or to patients with heavy irradiation pretreatment) have yielded similar favorable results in terms of relapse prevention *(111,113)*. However, treatment-related mortality after a busulfan-based conditioning regimen was higher than with the TBI-based regimen *(114)*. Several dose-escalation studies failed to show that more intensified conditioning regimens are capable of reducing further relapse incidence *(115)*.

Most studies and registry data indicate that transplantation in second remission is associated with an outcome superior to

that of chemotherapy *(100,102,116)*. With some exceptions, the results are definitively better in patients transplanted in complete remission (40–55%) than in those transplanted in partial remission or in relapse (20–25%) *(61,100,103, 107)*. Other important factors influencing outcome are the length of first remission, as well as high-risk features at diagnosis or at relapse *(54,104,106,108,118)*. Most of the studies documented in these publications, however, involve relatively small numbers of patients and none presents a comparison on a donor/no donor basis.

Few studies have compared the results of matched sibling-donor SCT in second remission with those of chemotherapy by matching variables associated with long-term outcome (*p*DFS at 5–8 yr after SCT, 40–62% vs 17–26% after chemotherapy alone) *(102,106,118)*. The comparison of 376 children who received a matched sibling transplant while they were in second remission with 540 relapsed children treated according to standard POG protocols showed a better DFS rate at 5 yr in the transplantation group (36% vs 16% in the chemotherapy group, $p < 0.001$). The results were independent of known prognostic factors indicating a high or low risk of relapse *(102)*. By using a matched-pair design, Borgmann, et al. demonstrated an advantage of SCT over chemotherapy for both early and late relapses (first remission duration < or > 36 mo). However, for late extramedullary relapses (including T-ALL), they also showed a very high probability of second continuous remission in patients not receiving allogeneic SCT *(61)*. Another German and Italian study demonstrated that early BM relapses (<6 mo after cessation of front-line therapy) treated with allogeneic SCT have an outcome similar to that of late relapses (*p*EFS at 7 yr, 69% vs 65%) *(108)* and superior to that with intensified rotational chemotherapy alone (*p*EFS at 3 yr, 33% vs 16%) *(108,118)*. However, according to the BFM experience in very early relapsing patients (especially those with T-cell ALL), the probability of DFS is nearly zero with chemotherapy alone, a finding that remains to be confirmed by further prospective studies *(21)*. For most patients who have an intermediate risk of further relapse, the lack of randomized studies makes it difficult to recommend allogeneic SCT unequivocally.

Two-thirds of ALL relapses occur in patients without a histocompatible sibling donor. This has recently led to an attempt to use alternative donors, including partially matched related donors, matched or partially mismatched unrelated donors, or cord blood donors *(119,120)*. Techniques such as depleting donor marrow of T-cells in order to prevent severe graft-versus-host disease (GvHD) have been effectively introduced in clinical practice *(121,122)*. Furthermore, the harvest of CD34 precursor cells from peripheral blood after cytokine stimulation is increasingly becoming the preferred approach, allowing the collection of a high number of hematopoetic precursor cells and a controlled infusion of donor lymphocytes *(123–125)*.

Only a few reports of studies on children undergoing SCT from partially related or unrelated donors have been published so far *(59,126)*. In a recently published matched-pair analysis, a high rate of treatment-related mortality (42%), but a lower relapse incidence (32%) than in autograft recipients (15 and 55%, respectively) were reported in patients with acute leukemia who lacked an HLA-identical sibling *(127)*. Morbidity and mortality have recently been dramatically reduced in the pediatric field *(59,128)*. The largest single center experience, with 50 ALL patients in second remission after previous chemotherapy according to MRC protocols, demonstrated an actuarial EFS at 2 yr of 53%, with no significant difference between the matched and mismatched group. The results were similar to the most favorable published reports for HLA-matched sibling SCT in relapsed ALL *(59,119,126,127)*.

5. AUTOLOGOUS STEM CELL TRANSPLANTATION

Autologous SCT is another approach that has been widely used in children in second remission *(118,129)*. The infusion of bone marrow or peripheral stem cells obtained by apheresis while the patient is in apparent remission has the advantage of being applicable to all patients in the absence of GvHD. To avoid the risk of reinfusing residual leukemic cells, several techniques have been used for treatment of bone marrow or peripheral blood in vitro with drugs, immunotoxins, or specific monoclonal antibodies *(130–133)*.

The results comparing autologous SCT with different postremission therapies are difficult to interpret and are not equivalent among the studies. DFS figures are in the range of 10–47% *(59,118,134)*. Again, only small numbers of patients are included in these series, and no randomized studies are published. In the recently published MRC UKALLR1 study and in a matched-pair analysis confined to patients with late relapse, no significant difference between autologous SCT (47% at 5 and 3 yr, respectively) and matched sibling-donor transplantation (53 and 45% at 5 and 3 yr, respectively) could be found *(59,135)*. An Italian study, in contrast, reported a 3-yr EFS estimate of 58% after matched sibling-donor SCT vs 27% in the autografted group *(118)*. Similarly, no advantage of autologous SCT over chemotherapy as postinduction treatment for children with ALL in second remission was demonstrated by the MRC experience (*p*EFS at 5 yr 47% for autografted patients and 48% for chemotherapy patients) *(59)* and another matched-pair analysis carried out by the BFM group (*p*EFS at 9 yr 32% for autografted patients vs 26% for chemotherapy patients) *(136)*. Also, Uderzo, et al. could not find any advantage of autologous SCT over chemotherapy in the postinduction treatment for children with late ALL relapse. The results were similar for early (17 and 20% *p*EFS, respectively) and late relapsing patients (41 and 55%, *p*EFS respectively) *(105)*.

Another retrospective Italian study, however, comparing the impact of autologous SCT in children with isolated early CNS relapse with that of conventional therapy, has demonstrated a favorable outcome at 5 yr in the autologous SCT group (56% vs 13% pEFS in the chemotherapy group) *(137)*. Good control of extramedullary disease by autotransplantation was also observed in other studies *(138,139)*. In a multivariate analysis, Billet, et al. found that the following characteristics favored a benefit from autologous SCT in childhood ALL in second remission: long duration of first remission, longer second remission duration before SCT, and a high number of infused nucleated cells per kilogram. With these criteria used to assign patients to therapy, treatment-related mortality decreased from 33% before 1986 to 5 or 6 % subsequently. Unexpectedly, initial prognostic factors such as leukocyte count lost their significance *(138)*.

Although autologous transplantation of hematopoetic stem cells lacks importance for most relapsed patients, the value of this approach for specific subgroups (i.e., those with early extramedullary relapses and very late bone marrow relapses) warrants further studies, which might profitably address the efficacy of continuation treatment after SCT (i.e., chemotherapy, immunotherapy, or molecular biotherapy, or a combination of these modalities) *(133,140,141)*. It is also expected that better monitoring of the chemotherapy response by using molecular techniques to detect minimal residual disease will facilitate the best treatment choice for the individual child with relapse.

6. CONTROVERSIAL ISSUES

6.1. Induction Failure: A Challenge for Innovative Treatment?

Although a second remission can be achieved in most children, relapsed ALL may show considerable drug resistance *(19,44,142)*. In about 10–20% of patients, remission cannot be induced (G. Henze, personal communication), or refractory leukemia develops during treatment. How to treat refractory ALL in children therefore remains a formidable challenge. The morbidity caused by further treatment may be intolerable, as most patients have already been exposed to intensive multiagent chemotherapy. Moreover, mortality caused by bacteremia and fungal infections in children with resistant disease is higher than in patients who respond to treatment *(143–145)*.

Allogeneic SCT may cure patients who never achieve remission with conventional chemotherapy. A study of 115 evaluable patients with refractory ALL recently showed a 98% remission rate with allogeneic SCT from HLA-identical siblings, and the 3-yr probability of leukemia-free survival was 23% *(146)*. In another study, 14 of 31 intensively pretreated children with ALL in first bone marrow relapse or refractory to initial therapy underwent SCT after remission induction with a combination of intermediate-dose cytarabine and idarubicin. After a median follow-up of 18 mo, seven of them (22%) were still in continuous remission *(147)*. The probability of survival is influenced by the remission status at the time of allogeneic SCT *(148)*.

In the emergency situation of treatment-resistant ALL, a suitable, HLA-matched sibling donor will be available for only 20–25% of children. Therefore, an urgent search for alternative sources of stem cells, including partially mismatched related donors or matched unrelated donors, is mandatory. With the development of national marrow donor programs throughout the world, the probability of identifying an unrelated donor has increased, and this procedure is now being widely applied in pediatric patients *(126,149)*.

Because time is a crucial factor in patients with ALL who fail to achieve a second remission and lack a matched sibling donor or a closely matched unrelated donor, a mismatched family member may represent an important stem cell source. For such patients, it has been shown that the transplantation of megadoses of haploidentical CD34+ cells is a realistic therapeutic option and an alternative to the use of unrelated cord blood *(122,150)*. However, engraftment failure and slow immunoreconstitution, leading to viral infections and early relapse, are major limitations of this transplantation source. Donor leukocyte infusion might ensure this goal and could be applied as therapy for relapsed ALL after allogeneic SCT or even for prophylaxis *(125,151)*.

The recent demonstration that an immunologically mediated graft-versus-leukemia effect plays a central role in delivering the antileukemic effect of an allograft has led to a fundamental reevaluation of the role of conditioning therapy in allogeneic transplantation. Recent animal studies have demonstrated that stem cell engraftment can occur with use of conditioning regimens that are minimally myelotoxic. This has prompted the development of less toxic nonmyeloablative conditioning protocols, the goal of which is to achieve donor stem cell engraftment, allowing the graft-versus-leukemia effect of the allograft to be exploited as the primary antileukemic strategy. Preliminary results using such nonmyeloablative clinical protocols confirm that durable engraftment of allogeneic stem cells can be achieved with a marked reduction in the immediate toxicity of transplantation. The extent to which this radically different transplant strategy allows the delivery of a sustained antileukemic effect is unclear at present, but it is likely that this approach will make an important contribution to allogeneic SCT strategies in the future *(152–157)*.

6.2. Incidence and Prognostic Relevance of Genetic Findings

There are only a few genetic findings with independent prognostic relevance. The most important are the chromosomal t(9;22), t(4;11), and t(12;21). Whereas the t(9;22) and t(4;11) markers confer a poor prognosis, qualifying such patients for high-risk treatment, t(12;21) is associated with a good prognosis, allowing patients to be treated in the low-risk arm of protocols.

It has not been clear whether patients with t(9;22) and infants with t(4;11) can be cured by either immunotherapy, novel chemotherapy, or SCT. For Ph-positive ALL, the outcome usually is poor and appropriate treatment is not known. Recently, in an international effort, data from 326 patients included in various therapy protocols were evaluated retrospectively. The overall survival probability at 4 yr was 38%, and the *p*EFS was 26% after front-line therapy. The initial prednisone response was recognized as the most valuable criterion for predicting the length of first remission (*p*EFS in good responders was 42%, compared with 9% in poor responders) *(65,66)*. Not surprisingly, the survival probability after relapse did not exceed 8–11% at 2 and 5 yr *(46,51)*.

A good response to prednisone was also reported to be a predictor of prolonged remission in infants with t(4;11)-positive ALL, who generally can expect a high rate of treatment failure (M. Schrappe, personal communication). Irrespective of age at diagnosis, the fast responder to steroid therapy tends to remain in longer continuous remission after front-line therapy. Whether the recently identified subgroup of Ph-positive or t(4;11)-positive ALL patients who show a good response to initial prednisone therapy *(66;* M. Schrappe, personal communication) can also expect a longer remission duration after retrieval therapy is not known. Generally, the presence of a cytogenetically occult t(12;21), reflecting the *TEL-AML1* fusion gene, is considered a favorable prognostic

factor *(158,159)*. This impression was challenged by reports of different incidences of this abnormality in populations of relapsed patients *(160,161)*. The unexpected equal frequency of *TEL-AML1* positivity in relapsed and newly diagnosed patiets observed by the BFM group contrasts with the relatively low rate of this abnormality in other series of relapsed patients. One explanation could lie in differences in the length of observation time, as relapses in *TEL-AML1*-positive patients usually occur late. Another explanation could reside in the selection of patients in relapsed cohorts, as populations with more late relapses would be expected to have an overrepresentation of *TEL-AML1*-positive patients. The third possible explanation can be found in the hypothesis that *TEL-AML1* fusion might itself represent a preleukemic genetic alteration and that antileukemic therapy might provide a second hit, transforming further cells that could generate a second leukemia in the following years. Thus, the incidence of TEL-AML-positive relapses could strongly depend on the type of chemotherapy used for first-line treatment *(40,162)*.

6.3. Is ALL Relapse Always an Indication for SCT?

6.3.1. Given a Fully HLA-Matched Sibling Donor, Is There a Reason to Exclude Patients with Late Marrow Relapses from SCT?

There is general agreement that the duration of first remission is a prognostic factor for relapsed ALL so late marrow relapse might not be an indication for allogeneic SCT. This opinion is not shared by all groups. Barret, et al. *(102)* compared the results of treatment with marrow transplants from HLA-identical siblings in 376 children (as reported to the IBMTR), with the results of chemotherapy in 540 children treated by POG. Interestingly, length of first remission of >48 or 60 mo was not associated with a benefit in favor of chemotherapy, contrary to the general assumption that late-relapse patients are not candidates for matched sibling donor transplantation in second remission *(60,104,117,118)*. One of the major objections against SCT in late-relapse children is that chemotherapy and radiation used for the preparation of SCT can induce rather severe late effects, especially in heavily pretreated patients *(163–166)*. As an example, Cohen, et al. examined factors that play a role in the final height achievement of patients who underwent SCT during childhood. Previous cranial irradiation and single-dose TBI produced the greatest negative effect on growth capacity. Fractionation of TBI reduces this effect significantly, and conditioning with busulfan and cyclophosphamide seems to minimize it *(167)*. As peritransplant mortality has decreased in the last 10 yr, an increasing advantage for SCT could be expected in experienced centers. For the time being, however, no definitive conclusion can be drawn regarding the best treatment for late-relapsing ALL because of the lack of prospective randomized trials.

6.3.2. Are genetically Fully Matched Unrelated Donors as Suitable as Matched Identical Sibling Donors for Patients with a Bone Marrow Relapse?

During the last few years, considerable improvements in the search for unrelated donors and in HLA typing and supportive care have contributed to higher survival rates after allogeneic stem cell transplantation from unrelated donors *(127,168,169)*. Only a few reports regarding children undergoing this type of SCT have been published so far *(59,126)*. The high rate of treatment-related morbidity and mortality in these children (42%), which was associated with a lower relapse incidence (32%), was recently shown to be dramatically reduced *(59,127,128)*. In a large single-center experience (256 ALL patients in second remission after previous chemotherapy according to MRC protocols), the actuarial EFS rate at 5 yr was 46%, with no significant difference between the matched related (45%) and matched unrelated (52%) groups *(59)*. These results are similar to the most favorable published reports for HLA-matched sibling SCT in relapsed ALL *(59,119,126,127)*. However, as long as not enough prospective investigations are available, matched unrelated donor SCT should be considered with caution in patients with a moderate risk for further relapse (e.g., in patients with late bone marrow relapses who have a realistic chance to survive without SCT).

6.3.3. Are There Candidates for Haploidentical SCT in Second Remission?

Patients without a realistic chance to stay in second remission with chemotherapy alone [including those with early bone marrow relapses, T-ALL relapses, Ph-positive ALL relapses, t(4;11)-positive relapses, and no HLA-matched family or unrelated donor] could benefit from haploidentical alloimmuno-cytotherapy, but the graft has to be depleted of T-cells. However, long-term immunosuppression renders those patients susceptible to severe viral infections, secondary lymphoproliferative disorders, and relapses. Several techniques that may overcome problems like adoptive immunotherapy, B-cell depletion, increasing doses of donor lymphocytes are under study *(150,170–172)*.

6.3.4. Is There a Place for "Mini" Transplantations in Second Remission ALL?

In view of the good results for patients who undergo SCT in second remission, more experimental transplantations should be reserved for patients in whom, for clinical reasons, transplant-related toxicity seems to exclude conventional conditioning regimen. So far, only preliminary experience exists in children to support experimental approaches with a nonmyeloablative regimen to overcome recurrent disease in ALL *(154)*.

6.4. Recommendations for Allogeneic SCT

It is widely accepted that for patients with very early bone marrow relapse (within 18 mo of achieving first remission), allogeneic SCT in second remission is the treatment of choice, if a histocompatible sibling is available *(102,106,108,118)*. In a recently published consensus paper, the EBMT-Pediatric Working Party considers the use of matched unrelated donors for patients relapsing during the first year of front-line treatment, if they lack a suitable family donor *(104,173)*. Emphasized in this group are T- ALL relapses and relapses with unfavorable cytogenetic features such as t(9;22) and t(4;11), because they lack a realistic chance of cure with conventional chemotherapy *(3,12,65)*.

Regarding patients with late bone marrow relapse (>36 mo after induction of first remission or after completion of maintenance therapy), data from the IBMTR have shown no benefit

from SCT and suggest chemotherapy alone for these patients *(174)*. In fact, most centers are reluctant to select patients relapsing 1 yr after cessation of primary treatment or later for allogeneic SCT *(60,117)*. Also, the EBMT-Pediatric Working Party in its consensus paper does not strictly recommend SCT from a compatible sibling donor if the relapse has occurred late, up to 4 yr from initial diagnosis, because a substantial number of patients may benefit from chemotherapy alone *(104,175)*. A recent retrospective analysis of the ALL-BFM relapse study, however, demonstrated that a peripheral blast cell count >1000/μL at relapse is associated with a dismal outcome in late bone marrow relapses, suggesting that these patients should be entered in an SCT program *(175)*.

Although the data on unrelated SCT for children are preliminary and include only a relatively small number of patients, many centers and the Pediatric Working Party of the EBMT have opened this approach for early relapsing patients (<6 mo off therapy), especially for those with a T-cell immunophenotype and t(9;22) or t(4;11) *(173,176)*.

6.5. Indications for Radiation Therapy in Relapsed Childhood ALL

6.5.1. CNS Irradiation for Patients with Isolated Bone Marrow Relapses

CNS irradiation has been replaced successfully for most patients with newly diagnosed ALL *(177,178)*. This has been achieved by intensification of CNS-directed therapy, including frequent IT injections and systemic administration of drugs with CNS activity (e.g., high-dose MTX or dexamethasone), resulting in a CNS relapse incidence of <6% *(6)*. Nevertheless, the CNS remains the most frequent site of recurrence after the bone marrow. In parallel with the development of CNS-directed therapy as a component of first-line treatment in most children with ALL, preventive cranial irradiation in patients with bone marrow relapse without obvious CNS involvement has substantially contributed to the success of salvage therapy *(83)*.

Intellectual impairment after treatment for relapses involving cranial irradiation has been reported mainly for preirradiated patients with CNS relapses who also had an increased exposure to IT therapy *(87,179)*. Therefore, before advocating the reduction or elimination of CNS radiotherapy for patients with isolated bone marrow relapses, one must keep the following considerations in mind: (1) that most of the patients will not have had radiotherapy before; (2) that extended exposure to drugs given intrathecally might also cause deficits; (3) that reports on neurologic sequelae mainly focus on test results in heavily irradiated children who mostly have had an acceptable quality of life; and (4) that radiation dose and age at the time of irradiation play a crucial role with respect to the risk of intellectual impairment. Furthermore, the risks of a subsequent relapse with the hazard of resistant disease or, if the disease is curable, a higher toll in late effects have to be considered carefully.

6.5.2. Local Irradiation for Testicular Relapse

In patients with isolated testicular relapses, systemic retreatment and local radiotherapy generally are applied to one or both affected testes. This practice has been questioned, since in a small series of patients systemic chemotherapy including high-dose MTX led to sustained second remissions without testicular irradiation *(31)*. This result is of interest because radiotherapy with a common dose of 24 Gy will render most patients in need of hormone replacement treatment *(180)*. Avoiding local radiotherapy, especially in late testicular relapses, could be an attractive alternative worthy of further investigation in a larger number of patients. However, the intensity of first-line treatment, time to relapse, and speed of response to the salvage therapy should be kept in mind whenever a controlled study is being considered. It also remains to be shown in larger series whether response kinetics, measured by detection of minimal testicular disease, might contribute to the decision.

6.6. Recommendations for Radiotherapy (BFM Strategy)

6.6.1. Preventive CNS Therapy

It appears that 18 Gy given in a prophylactic attempt to the cranium, together with systemic and triple IT therapy, may be sufficient to prevent CNS relapse. In preirradiated patients, the cumulative radiation dose should not exceed 24 Gy *(53,58)*. If the previously delivered dose exceeded 24 Gy (18 Gy in children younger than 2 yr) or 18 Gy (15 Gy in children younger than 2 yr), and the interval to the previous irradiation was <24 mo, the replacement of radiotherapy by intensified intrathecal therapy should be considered *(58)*.

6.6.2. CNS Relapse Therapy

If the previously delivered radiation dose was >18 Gy (15 Gy in children younger than 2 yr), irradiation with 15 Gy should be considered. A dose reduction to 15 Gy is also warranted in case the interval from the primary irradiation was <24 mo. Children younger than 2 yr previously irradiated with 15 Gy should receive only 12 Gy (G. Henze, personal communication). To avoid a possibly higher risk for the development of a subsequent CNS relapse, an intensified IT treatment has to be considered *(21,83,85,181)*.

6.6.3. Testicular Relapse Therapy

In case of disease manifestation, a radiation dose of 18 Gy is recommended. If the biopsy is negative, 15 Gy should be enough. If the involved testis has not been removed or if the manifestation of the relapse is bilateral (isolated or combined), 18 Gy should be used *(58)*.

6.7. How Can the Detection of Minimal Disease Influence Treatment Strategies?

6.7.1. Could Early Diagnosis and Treatment of an Imminent Relapse Influence Prognosis?

Cytomorphology defines both remission and relapse. Lowering the threshold level for detection and monitoring of minimal residual disease by use of more sensitive molecular genetic or immunophenotypic methods, and modifying therapy according to these results, is a intriguing concept under investigation in clinical trials for newly diagnosed diseases *(24,182–185)*. Since the leukemic cell burden is a well-accepted prognostic factor in newly diagnosed ALL, the early disappearance of leukemic cells and early detection of a relapse might also be attractive prognostic criteria for relapsed disease *(186)*.

There are, however, three objections to the use of molecular genetic minimal residual disease screening to detect immu-

noglobulin or T-cell receptor gene rearrangement: (1) depending on the screening methodology used, clonal evolution might produce a considerable proportion of false-negative results *(187–190)*; (2) the time between molecular genetic detection of an emerging leukemic clone and cytomorphologic evidence of relapse might be as short as 1–2 wk, thus putting the clinical utility of the first approach into question; (3) with more sensitive PCR methods, a clinically significant lower threshold level remains to be defined, primarily because detection of minimal residual DNA or RNA below a certain level might not always mean residual disease. Whether immunologic detection of minimal disease with flow cytometry would meet clinical requirements more accurately remains to be shown in larger series *(191–193)*.

6.7.2. Could Monitoring of Minimal Disease Kinetics During Salvage Chemotherapy Influence Relapse Therapy?

Minimizing the tumor load before SCT is a task to be met. Correlation between the amount of pretransplant tumor load and the risk of posttransplant relapse has been shown repeatedly *(194–196)*. The standard conflict between the chemotherapist and the cytotherapist is emphasized by the following issue: whether the pretransplant toxicity burden influences the risk of transplant failure or whether insufficient chemotherapy before transplantation leads to an increased risk of relapse. This question could be partly solved by pretransplant chemotherapy guided by minimal residual disease. Still, data will have to be interpreted in the context of the quality and intensity of relapse therapy applied, and these data are not yet available.

6.8. Definition of Risk Groups (BFM Strategy)

Tailoring of therapy according to biologic and clinical risk factors is a common tenet in the management of newly diagnosed ALL patients. Systematic approaches toward identification of adverse prognostic features indicating a favorable or unfavorable outcome in patients after relapse have recently created a basis for prospective stratification into different risk groups. The BFM group was the first to address the effect of tailored therapy systematically in five consecutive multicenter studies (ALL-REZ BFM 83, 85, 87, 90, and 96) *(21,53,54;* G. Henze, personal communication). Consideration of all risk factors with an independent influence on outcome has led to the identification of four treatment groups:

1. *Therapy group A (S1)*: all patients with late (>6 mo off therapy) extramedullary relapse (*p*EFS, 77%).
2. *Therapy group B (S2)*: all patients with very early (<18 mo after initial diagnosis) and early (between 18 and 30 mo after diagnosis) extramedullary relapse, all combined (early and late) non-T-ALL relapses, and late non-T-ALL bone marrow relapses (*p*EFS, 35%).
3. *Therapy group C (S3)*: all patients with early isolated non-T-ALL bone marrow relapse (*p*EFS, 4%).
4. *Therapy group D (S4)*: all patients with very early combined and isolated bone marrow relapse, and all patients with T-cell bone marrow relapse (remission rate, only 50–60%; median remission duration median 3 mo).

If accepted internationally, this stratification, based on retrospective findings in various study groups (BFM, MRC-UKALL, CCG), would provide a common valuable base for comparison in future prospective and randomized studies.

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14 Treatment of Relapsed Acute Lymphoblastic Leukemia

Perspective 2

GÜNTER HENZE AND AREND VON STACKELBERG

CONTENTS

1. INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most frequent malignant disease in childhood. With currently used treatment, event-free survival rates (EFS) range from 70 to 75%. However, relapse of ALL remains the fourth most frequent diagnosis in childhood cancer, with an incidence ranging close to that of neuroblastoma *(1,2)*. Compared with the prognosis for newly diagnosed ALL, the chance of long-term survival is substantially reduced in cases of relapse. The remission rate is limited by a higher rate of induction deaths owing to reduced tolerance to treatment after organ-toxic frontline therapy. Furthermore, the rate of nonresponses to treatment is increased, since blast cells surviving the intensive front line treatment have had the chance to develop resistance toward commonly used antileukemic drugs. Even if a complete remission (CR) can be achieved, the rate of subsequent relapses is high.

The probability of long-term survival can be predicted from a variety of prognostic factors. Recent research focuses on the detection of additional prognostic parameters that might help to stratify patients for assignment to treatment regimens of adequate intensity, which include (besides multiagent chemotherapy) different modes of stem cell transplantation (SCT). Usually, intensified polychemotherapy containing high-dose elements is used for induction of a second CR. Depending on their particular set of risk factors, patients may have a reasonable chance to remain in continuous CR after conventional intensive consolidation chemotherapy and maintenance therapy or may require further treatment intensification by SCT. For patients with an intermediate prognosis after chemotherapy alone, the indication for SCT remains controversial.

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1.1. DIAGNOSTICS

ALL relapse is defined as the reappearance of lymphoblastic leukemic cells in any anatomic compartment following CR induction. As in newly diagnosed ALL, the diagnosis of relapsed ALL must be unequivocal, requiring a complete clinical workup to detect clinically overt and occult manifestations of leukemia. Besides careful physical examination, this includes bone marrow (BM) aspirations (from several sites if necessary), a BM biopsy in cases of *punctio sicca*, a diagnostic lumbar puncture, and appropriate local imaging for other suspicious manifestations. The leukemic cells should be characterized by conventional morphologic and immunophenotyping, as well as cytogenetic and molecular genetic procedures. Only this comprehensive information together with clinical findings allows one to classify the leukemic subtype adequately and to assess the prognosis of individual patients *(3)*.

1.1.1. Morphology

The generally accepted diagnostic method is simple light microscopy with standard staining according to Pappenheim or Wright and morphologic classification according to criteria of the French–American–British (FAB) Cooperative Group *(4,5)*. An isolated BM relapse of ALL is defined as >25% lymphoblastic leukemic cells in the BM smear without evidence of leukemia at other sites. The diagnosis of BM involvement in cases of proven extramedullary relapse requires the presence of

at least 5% BM blasts. The most common sites of extramedullary manifestations are the central nervous system (CNS) and the testicles. A CNS involvement has to be verified or excluded in all patients. It is defined as the presence of at least 5 white blood cells/μL in the cerebrospinal fluid (CSF) with blast cell morphology on cytospin preparation. A testicular relapse is strongly suspected in cases of unilateral or bilateral painless testicular enlargement. It has to be confirmed by biopsy (or orchiectomy) of the involved testicle(s), and involvement of a contralateral, not enlarged testicle has to be excluded. Infrequently, a variety of other extramedullary sites may be involved, such as the skin, bone and muscle, abdominal organs, or eye. Cytochemical staining for acid phosphatase, periodic acid-Schiff reaction, peroxidase, and esterase can help to discriminate ALL relapse from secondary acute myeloid leukemia (AML) *(6)*.

1.1.2. Immunophenotyping

Flow cytometry with a standard panel of B-cell, T-cell, and myeloid markers is necessary to confirm the diagnosis and to determine the immunophenotype, i.e., B-cell precursor or T-cell ALL.

1.1.3. Genetics

Genetic aberrations of the leukemic cells at relapse should be analyzed, repeating the diagnostic panel used at first manifestation. This includes quantitative DNA analysis for ploidy (DNA index) *(7)*, cytogenetic analysis *(8,9)*, and screening for relevant translocations by molecular genetic methods. Furthermore, clone-specific rearrangements of T-cell receptor and immunoglobulin genes can be used as markers to monitor patients for minimal residual disease *(10)*. Several genetic aberrations have been shown to be of prognostic relevance in children with ALL in relapse, including the translocations t(9;22) and t(12;21) *(11,12)*.

1.2. Controversies in Diagnostics

1.2.1. Bone Marrow Involvement in "Isolated" Extramedullary Relapse

Commonly, BM involvement is diagnosed if there are at least 5% BM blasts in cases of cytologically or histologically proven extramedullary relapse. Because immunophenotyping and molecular genetic methods are available, lymphoblastic leukemic cells can be detected far below the threshold of conventional light microscopy, which is 1–5/100 nucleated BM cells. Recently, occult BM involvement has been demonstrated in a substantial number of patients with "isolated" extramedullary relapse *(13,14)*. These findings confirm clinical experience indicating that isolated extramedullary relapse has to be regarded as a systemic disease likely to progress to clinically overt systemic relapse. Consequently, besides local treatment, intensive systemic therapy needs to be administered and may abolish the prognostic relevance of occult BM involvement in "isolated" extramedullary relapse, because even overt involvement of the BM in extramedullary relapse lacks prognostic relevance, as has been reported by the Berlin–Frankfurt–Münster (BFM) Relapse Study Group *(15,16)*.

1.2.2. Subclinical Isolated CNS Relapse

Serial therapeutic lumbar punctures performed during frontline maintenance therapy can detect CNS relapses at a very early stage before clinical symptoms become apparent. Exceptionally favorable treatment results have been reported for such patients *(17)*. In other groups, including the BFM Study Group, lumbar punctures beyond the intensive frontline treatment period are not routinely performed, and CNS relapses are usually diagnosed on the basis of clinical symptoms, including headache, vomiting, and/or neurologic deficiencies. In most of these cases, a clear blast cell pleocytosis can be found. Rarely, magnetic resonance imaging may be necessary to prove or rule out CNS leukemia. It remains open whether (in the absence of treatment) blasts in the CSF at a subclinical stage would always progress to clinically overt CNS relapse and subsequent BM involvement or might disappear without generating a clinically relevant relapse. Furthermore, in cases of chemical arachnoiditis after serial therapeutic lumbar punctures, lymphatic cells in the CSF may be misinterpreted as a CNS relapse. Therefore, in our opinion, an isolated CNS relapse should be diagnosed only when there are clear clinical symptoms and at least 5 white blood cells/μL of CSF with blast cell morphology. Lymphatic pleocytosis with single suspicious cells is not sufficient for the diagnosis of an isolated CNS relapse, although this finding does warrant lumbar puncture repeated after 1 or 2 wk *(1)*.

1.2.3. Subclinical Testicular Relapse

Several groups have evaluated the significance of routine testicular biopsies at elective cessation of frontline therapy. The rate of positive biopsies in these studies ranges from 4 to 10% *(18,19)*. The authors report a worse prognosis for boys with early diagnosis of occult testicular relapse, compared with those who have late, clinically overt testicular relapses. Thus, early occult leukemic infiltration of the testes seems to represent aggressive minimal residual disease, which may progress to an early overt testicular relapse. However, the treatment results for boys with early occult isolated testicular relapse are not superior compared with those for boys with overt early isolated testicular relapse. Furthermore, routinely performed testicular biopsies cannot predict late testicular relapses *(19–21)*. Since routinely performed testicular biopsies have not contributed to improved treatment results, they have been abandoned by most study groups.

2. BIOLOGIC AND CLINICAL CONSIDERATIONS

2.1. Evolution of Leukemic Cells from First Manifestation to Relapse

The evolution of leukemic cells from first manifestation to relapse and specific changes at relapse are topics of intensive investigation. Morphologic changes of the FAB subtype as well as intralineage shifts of the immunophenotype have been described *(22–24)*, and loss or acquisition of myeloid antigens has been observed *(25)*. These findings remain without clinical relevance.

More interestingly, the changes in genetic features raise questions about the reliability of clone-specific markers for monitoring of minimal residual disease. A variety of clonal evolution phenomena have been described. Because clonal diversity has been found in leukemic cells at first diagnosis, often being associated with an inferior prognosis, clonal evolution detected at relapse could be explained by selection of one of the initial clones or by completion or modification of

preexisting rearrangements *(26–30)*. Clonal stability in rearrangements of at least one parameter, T-cell receptor δ,γ or Ig heavy chain, has been found in leukemic cells from patients who relapsed more than 5–10 yr after diagnosis, disproving the hypothesis that late ALL relapses are secondary leukemias *(31,32)*. It is recommended that more than one gene locus be used as a clonal marker for the detection of minimal residual disease to overcome the problem of clonal diversity and clonal evolution *(10)*. Several mechanisms may lead to resistance against antileukemic drugs during the evolution from newly diagnosed ALL to ALL in relapse, as discussed later in this chapter.

2.2. Prognostic Factors: Established, New, and Future

Time from CR induction to relapse, site of relapse, and immunophenotype are well-established prognostic factors. Additionally, the leukemic tumor burden as measured by the peripheral blast cell count (PBC) and the translocation t(9;22), or its molecular-genetic equivalent, *BCR/ABL*, are reported to be relevant prognostic factors in relapsed ALL. Further possible prognostic parameters such as the translocation t(12;21), corresponding to the fusion transcript *TEL/AML1*, the dynamics of response to treatment, persistence of minimal residual disease, and results of in vitro drug resistance analyses await confirmation by ongoing or future trials. However, whereas a broad diagnostic panel allows one to determine a variety of relevant prognostic factors, the panel of established therapeutic options is restricted to a second course of polychemo-therapy, possibly followed by SCT, and is limited by treatment-related toxicity.

2.2.1. Time to Relapse

The most evident prognostic factor is the time to relapse, defined as the duration of CR prior to relapse *(2,33)*. In some groups, time to relapse is related to the duration of remission after elective cessation of frontline treatment *(34)*. Clinical trials have shown that a prolonged maintenance therapy can delay relapse and that the duration of antileukemic treatment is a relevant prognostic criterion *(35,36)*. When the results of clinical trials are compared with respect to time to relapse, the different definitions of this particular end point must be taken into consideration *(1)*.

Some authors define early or late relapse as relapse occurring during frontline therapy or off therapy, respectively. Other groups define early as before 18–24 mo after the first diagnosis of ALL and late as later than 24–36 mo after diagnosis. According to the experience of several consecutive trials, the BFM Study Group defined time to relapse as very early (within 18 mo after initial diagnosis), early (beyond 18 mo after initial diagnosis up to 6 mo after cessation of frontline treatment), and late (beyond 6 mo after cessation of frontline treatment). The event-free survival (EFS) of 910 patients treated between 1983 and 1997 from trials ALL-REZ BFM 83, 85, 87, 90, and 95 according to these categories is shown in Fig. 1. An early relapse is associated with a higher rate of nonresponse to treatment, a shorter duration of second CR, and a lower relapse-free survival rate. Similar results have been described by other groups *(2,37,38)*.

2.2.2. Site of Relapse

Site of relapse has been shown to be a significant prognostic factor in childhood ALL. Children with extramedullary relapses have a better prognosis than those with an isolated BM relapse. Extramedullary relapses are thought to originate from leukemic cells that survive frontline treatment in an extracompartmental sanctuary. Insufficient exposure to chemotherapeutic agents may provide favorable conditions for the development of drug resistance. The microenvironment of the CNS and the testes supports a slow growth rate of leukemic cells and protects vulnerable cells from external influences *(39–41)*. Therefore, manifestations of leukemia in such sanctuaries require specific local treatment. Systemic subsequent relapses occur frequently, demonstrating the necessity of additional intensive systemic treatment *(2,36,42–44)*. These findings are underlined by the presence of occult BM involvement in children with "isolated" extramedullary relapse, detected by more sensitive methods such as multiparameter flow cytometry or molecular genetics *(14,45,46)*.

Interestingly, children with a combined BM relapse have been reported to have a superior prognosis compared with that of children who have an isolated BM relapse *(16)* (Fig. 2). Some authors believe this prognostic discrepancy is related to a generally longer duration of first remission in patients with combined compared with isolated BM relapse *(2,47)*; however, the outcome in the former subgroup remained superior after adjustment for time to relapse *(16)*. Furthermore, the EFS of children with extramedullary relapse is not influenced by the degree of concomitant leukemic metaplasia in the BM. These findings suggest that in combined relapses, the BM blasts derive from leukemic cells that have reseeded the marrow from an extramedullary compartment rather than representing the site of origin of systemic relapse *(48)*. The prognostic relevance of occult BM leukemia in cases of an "isolated" extramedullary relapse remains open and requires further prospective evaluation.

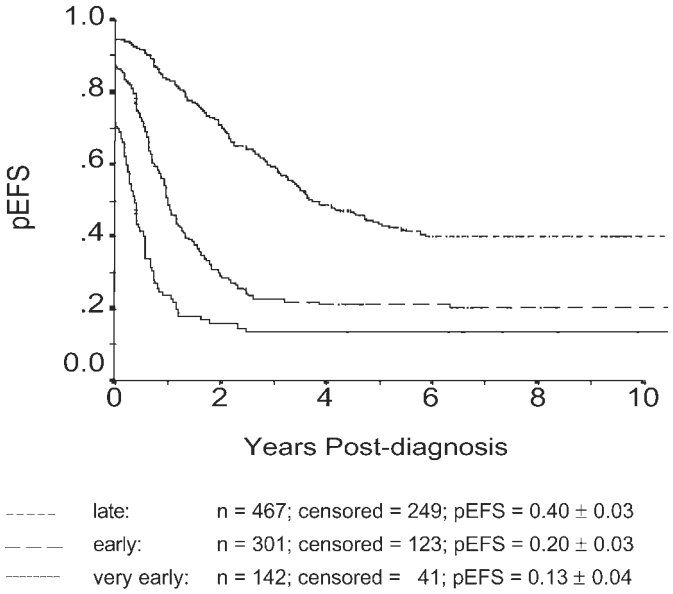

Fig. 1. Event-free survival probability (pEFS) for children with ALL relapse according to time to relapse (SCT censored; log-rank test, $p < 0.001$). Trials ALL-REZ BFM 83–95.

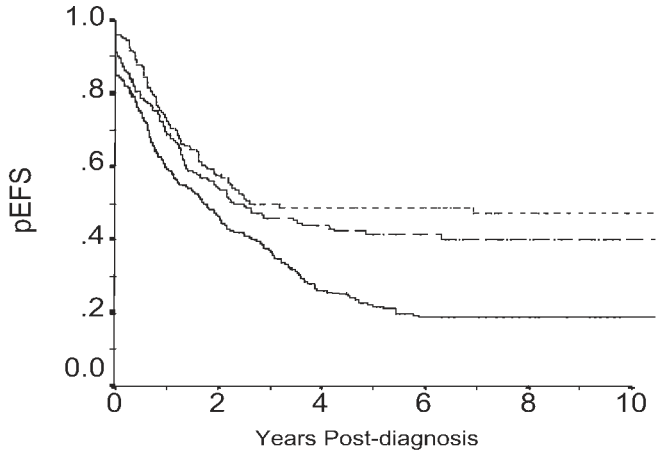

Fig. 2. Event-free survival probability (pEFS) for children with ALL relapse according to site of relapse (SCT censored; log-rank test, $p < 0.001$). Trials ALL-REZ BFM 83–95.

Table 1
Frequencies of Immunophenotypes in Children with a First ALL Relapse from Trials ALL-REZ BFM 83, 85, 87, 90, and 95[a]

Lineage	*Maturity*	*No.*	*%*
T-cell	Pre-T	21	1.8
	Mature T	139	11.8
B-cell precursor	Pro-B	69	5.9
	CALLA	710	60.5
	Pre-B	154	13.1
BAL (biphenotype)		9	0.8
No data		72	6.1
Total		1174	100.0

Abbreviations: BAL, biphenotypic acute leukemia; CALLA, common ALL antigen.
[a]Including pilot studies.

2.2.3. Immunophenotype

ALL is generally classified as having B-cell precursor or T-cell origin. An additional consideration is the presence of myeloid markers, which have been found to be associated with the *TEL/AML1* fusion transcript *(49,50)*. Frequencies of the immunophenotypes in children with relapsed ALL treated according to trials ALL-REZ BFM 83–96, including pilot studies, are given in Table 1. About 13% of the relapsed cases have a T-cell immunophenotype, which has been reported to be a significant adverse prognostic factor *(22,33,51)*. Relapses of T-cell ALL occur after a relatively short first CR. The high rate of nonresponse to salvage treatment suggests that the cells are highly drug resistant. The duration of second CR, if achieved, is very short. The stage of maturation of the T-cell and B-cell precursor lineages, as well as the presence of myeloid markers, has no prognostic relevance.

2.2.4. Independent Prognostic Relevance of Established Risk Factors: Proposal for a Comprehensive Risk Group Classification

Time to and site of relapse as well as immunophenotype are highly significant prognostic factors in univariate and multivariate analyses (Table 2). Based on these findings, patients of the ongoing trial ALL-REZ BFM 96 are allocated to one of four strategic groups, S1–S4 (Table 3). In retrospective analyses, EFS ranges from a favorable (S1) to a dismal prognosis (S3 and S4; Fig. 3). Patients in group S2 have an intermediate prognosis, with an EFS rate of 35% at 6 yr. For this heterogeneous group, further prognostic features may be useful in identifying patients with an acceptable prognosis after conventional chemotherapy and those requiring intensification treatment such as SCT.

2.2.5. Peripheral Blast Cell Count: A Measure of Tumor Burden

The PBC count, an indicator of tumor burden at relapse, has been identified as an additional prognostic factor for children with late, isolated non-T-cell BM relapses, who represent the largest subgroup of S2 patients. Whereas children with no detectable blasts have a more favorable prognosis, the probability of EFS at 5 yr is <20% in those with a PBC count >10,000/μL *(52)*.

2.2.6. Are Genetic Alterations Suitable Prognostic Factors?

At first diagnosis of ALL, the Philadelphia chromosome, corresponding to the translocation t(9;22) or its molecular equivalent, the fusion transcript *BCR-ABL*, can be detected in 2.3–3.6% of children. It is associated with a poor prognosis and a high relapse rate *(53–55)*, so that most authors recommend SCT in first CR for this patient subgroup *(56,57)*. However, a good response to initial cytoreductive treatment with prednisone has been reported as a suitable criterion to define a *BCR-ABL*-positive subgroup with an acceptable outcome after conventional chemotherapy *(58)*.

About 10% of ALL patients who relapse have *BCR-ABL*-positive disease *(11)*. This subgroup has an extremely poor prognosis, with an EFS probability of <10% at 2 yr. *BCR-ABL* positivity is associated with adverse risk factors, such as short duration of first CR and high PBC counts. However, in analyses matching these cofactors, as well as in multivariate analyses, *BCR-ABL* expression proved to have independent prognostic significance *(11)*.

More recently, the cryptic translocation t(12;21)(p13;q22) with the resulting fusion transcript *TEL-AML1* has been described as the most frequent genetic aberration in childhood B-cell precursor ALL. At first diagnosis, *TEL-AML1* can be found in about 25% of patients. Positivity for this fusion gene indicates a favorable prognosis and a low relapse rate *(59–61)*, and some authors have described a low or similar rate of *TEL-AML1*-positive patients at relapse of ALL *(12,62,63)*. Expression of *TEL-AML1* at relapse is associated with a long duration of first CR, and such patients have a significantly better EFS rate after second CR induction than do patients without this marker *(12)*. However, subsequent relapses can occur, again after relatively long periods of second CR *(64,65)*. The prog-

Table 2
Multivariate Cox Regression Analysis of Event-Free Survival (Studies ALL-REZ BFM 83–95) According to Relapse Category[a]

Factor	*Category*	*Risk ratio*	*95% CI*	*p value*
Time point	Late	1.0	(Reference group)	
	Early	3.7	3.0–4.5	<0.001
	Very early	5.9	4.7–7.4	<0.001
Site	Isolated extramedullary	1.0	(Reference group)	
	Combined	2.0	1.5–2.7	<0.001
	Isolated BM	3.5	2.7–4.5	<0.001
Immunophenotype	B-cell precursor	1.0	(Reference group)	
	(Pre-)T-cell	2.3	1.8–2.8	<0.001

Abbreviations: CI, confidence interval; BM, bone marrow.
[a]Cox regression model: $n = 1102$; 642 events; χ^2 (Wald) = 443.7; $p < 0.001$.

Table 3
Stratification Groups S1–S4 of Trial ALL-REZ BFM 96 Defined by the Risk Factors Time, Site, and Immunophenotype of Relapse

	B-cell precursor			*(Pre-) T-cell*		
Time point	*Extra-medullary*	*Combined BM*	*Isolated BM*	*Extra-medullary*	*Combined BM*	*Isolated BM*
Very early	S2	S4	S4	S2	S4	S4
Early	S2	S2	S3	S2	S4	S4
Late	S1	S2	S2	S1	S4	S4

Abbreviations: BM, bone marrow.

nostic significance of *TEL-AML1* has to be evaluated carefully on the basis of conclusive long-term results before reductions in treatment intensity, as suggested by some authors, are attempted in clinical trials.

2.3. Drug Resistance: Origins, Prognostic Relevance, and Therapeutic Implications

Response to treatment at relapse is much inferior to that in newly diagnosed ALL, partly because of a negative selection of patients with drug-resistant disease. Drug resistance can be acquired either by selection of subclones with a primary capacity for the development of drug resistance *(66)* or as a consequence of exposure to antileukemic drugs *(67)*. The best known mechanism of multiple drug resistance (MDR) is regulated by P-glycoprotein, functioning as a cellular drug efflux pump. The presence of MDR has been associated with an adverse prognosis. At relapse, the proportion of patients with P-glycoprotein expression is increased, and a trend toward a poorer prognosis owing to this marker has been described *(68)*, although no relevant induction of P-glycoprotein expression of initially negative patients has been found at relapse *(69)*. However, among primarily positive patients, an increased function of P-glycoprotein at relapse has been reported *(70)*. Attempts to inhibit P-glycoprotein function by drugs such as verapamil or cyclosporin-A have been largely unsuccessful for clinical use *(71,72)*. Other MDR mechanisms have been described, including the expression of glutathione transferase and metallothionein. Whereas the prognostic relevance of these single markers remains questionable, a synergistic interactive effect was noted when they were expressed together *(73)*.

Specific mechanisms have been discovered concerning resistance against methotrexate (MTX), namely, an increased activity of dihydrofolate reductase (DHFR), impaired methotrexate membrane transport, and impaired methotrexate polyglutamylation *(74,75)*. The amplification of the *DHFR* gene leads to an increased intracellular metabolization of MTX *(76,77)*, which in turn causes resistance against this agent by a

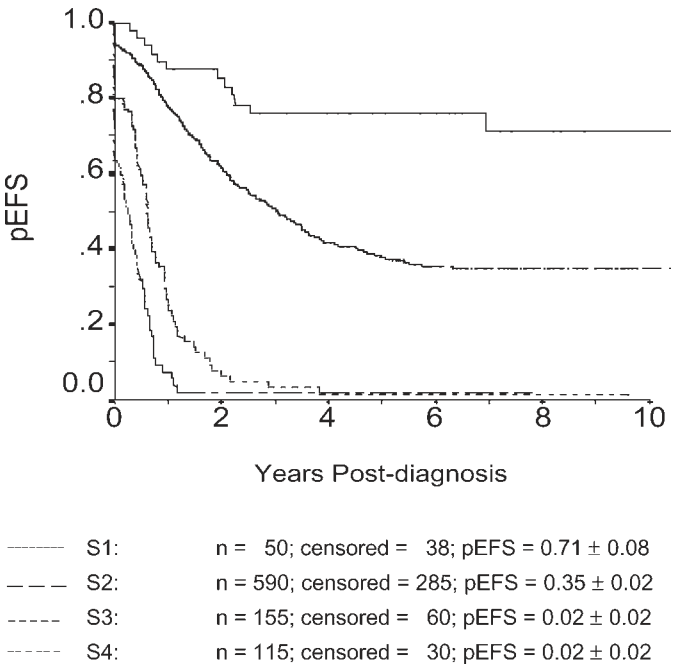

Fig. 3. Event-free survival probability (pEFS) for children with ALL relapse of stratification groups S1–S4 (SCT censored; log-rank test, $p < 0.001$). Trials ALL-REZ BFM 83–95.

decreased intracellular polyglutamylation of the drug. The activity of the MTX-polyglutamate-synthesizing enzyme folylpolyglutamate synthetase has been found to be twofold lower in T-lineage compared with B-lineage ALL *(78)*. This might explain the higher MTX resistance of leukemic T-cells compared with B-cell precursors *(79)*. MTX resistance can be partly circumvented in vitro by novel antifolates such as trimetrexate, which enters the cells independently of an active folate transport system, and by higher drug concentrations or longer durations of exposure to the drug *(80–82)*.

Induction of apoptosis in lymphoblastic leukemia cells is an elementary tenet of antileukemic treatment. Thus, loss of capacity to commit apoptosis is an important mechanism of resistance to treatment. A variety of genes or rather their gene products are known to be involved in the mediation and modulation of apoptosis. The tumor suppressor gene *p53* drives the cell cycle toward apoptosis after chemotherapy- or radiation-induced DNA damage *(83)*. Therefore, disabling mutations and deletions of *p53* are associated with aggressive and treatment-resistant malignant diseases.

The role of *p53* in childhood ALL remains controversial. Some authors found an association between *p53* mutation and a poor prognosis at first diagnosis *(84)*. Additionally, the function of *p53* is linked to a variety of regulatory proteins. Overexpression of the *MDM2* gene product inhibits *p53* function and consequently decreases the capacity for apoptosis *(85)*. In primary ALL, it is associated with early relapse and anthracycline resistance *(86–88)*. New *p53* mutations have been identified at ALL relapse, but they lacked prognostic impact *(89)*. The BCL2 protein is known to inhibit apoptosis, although overexpression of BCL2, in childhood ALL has not shown prognostic relevance *(90,91)*. Interaction of *p53*, BCL2 and BAX, another apoptosis-modulating protein, has been observed without conclusive results concerning prognostic relevance and therapeutic consequences *(92,93)*.

An important tool to assess the drug resistance of ALL cells is the methyl-thiazol-tetrazolium (MTT) assay, which measures the viability of leukemic cells after exposure to a panel of antileukemic drugs in vitro. A significantly higher resistance to glucocorticoids, L-asparaginase, anthracyclines, and thiopurines has been observed at relapse compared with first diagnosis of childhood ALL *(67)*. The most prominent differences have been observed for glucocorticoids, when a 24-fold higher dose was required to achieve an antileukemic effect similar to that seen in nonresistant cases. Resistance to glucocorticoids is linked to quantity and function of the glucocorticoid receptor and to postreceptor pathways leading to the induction of apoptosis *(94)*. Future approaches in the treatment of children with ALL relapse might include attempts to circumvent or modulate glucocorticoid resistance.

Furthermore, resistance to anthracyclines could be modulated in vitro by several agents. Cyclosporin A and verapamil, which have been shown to sensitize leukemic cells to anthracycline treatment, might be of value in individual patients, if a sensitizing effect could be demonstrated in vitro *(72)*.

The BFM Relapse Study Group performed two consecutive pilot studies (ALL-REZ BFM P91 and P92) for patients with a very poor prognosis. Treatment consisted of polychemotherapy courses individually designed to include drugs with the best antileukemic activity according to in vitro results of the MTT assay. Although this strategy proved feasible, the results were not superior to those for historical controls, and the toxicity was even higher than with conventional approaches *(95,96)*.

3. TREATMENT STRATEGIES

Since the early 1970s, prospective attempts have been made to treat children with ALL in relapse, mostly using regimens that correspond to frontline therapy. Although second remissions could be induced, the remission rates have been unsatisfactory, indicating an obvious need for more intensive treatment to overcome the drug resistance of cells that had survived the first round of chemotherapy *(97–100)*. A variety of different drugs and drug combinations have been used for second induction, consolidation, and maintenance therapy. Despite the increasing intensity of induction and postremission therapy, the rate of subsequent relapses was high, leading to the introduction of SCT as an alternative postremission treatment *(101,102)*. It is somewhat problematic to draw conclusions from published data about the treatment of children with relapsed ALL and to compare results from different studies. Frontline protocols have become increasingly intensive over time, so that recent relapse trials have enrolled very heavily pretreated patients, whose leukemic cells are likely to be much more resistant to chemotherapy than those treated with the less intensive frontline regimens of the 1970s. Furthermore, the heterogeneity of risk stratification among different study groups makes a comparison of treatment results difficult. For instance, time to relapse, or the duration of first CR, is the most relevant prognostic factor for relapsed ALL, but definitions of early and late relapse vary widely in different trials and published reports.

Many of the published studies comprise only low patient numbers. Whereas frontline treatment was well standardized in most study groups, salvage treatment was frequently open to individual and experimental approaches of single centers. Therefore, published results frequently refer to a patient population accrued during a defined time period but having received heterogeneous treatment. Table 4 summarizes treatment results for patients with BM relapse defined by site of origin and time to relapse. Although these reports are based on comparably large patient series, most do not include a detailed description of the treatment used *(2,37,38)*.

3.1. Chemotherapy

3.1.1. Reinduction Therapy for Bone Marrow Relapse

Table 5A,B displays an overview of results with published reinduction regimens in children with relapsed ALL. Remission rates for children with BM relapses range from 75 to 100%, depending on the time to relapse and size of the patient cohort *(35,103–111)*. In most published studies for children in first relapse, reinduction therapy consisted of a 4-wk regimen with prednisone, vincristine, and an anthracycline—mostly daunorubicin, often supplemented by L-asparaginase. In a large study of the Pediatric Oncology Group (POG), a second CR in patients with early BM relapse (i.e., before 6 mo after completion of frontline therapy) was induced with a 4-wk schedule of prednisone, vincristine, daunorubicin, and L-asparaginase

Table 4
Treatment Results for Patients with BM Relapse According to Time and Site of Relapse

			Time to relapse					
			Early		*Intermediate*		*Late*	
Author	*Protocol*	*Site*	*No.*	*Outcome*	*No.*	*Outcome*	*No.*	*Outcome*
Schroeder et al., 1995 *(37)*[a]	NOPHO 1981–94	Isolated + combined	114	EFS (8 yr) = 8%	42	EFS (8 yr) = 19%	60	EFS (7 yr) = 50%
Gaynon et al., 1998 *(2)*[b]	CCG 100 series 1985–89	Isolated	233	DFS (6 yr) = 5%	194	DFS (6 yr) = 10%	215	DFS (6 yr) = 33%
		Combined	34	DFS (6 yr) = 9%	26	DFS (6 yr) = 11%	60	DFS (6 yr) = 48%
Wheeler et al., 1998 *(38)* [a]	UKALL X 1985–93	Isolated	83	DFS (5 yr) = 1%	29	DFS (5 yr) = 14%	123	DFS (5 yr) = 33%
		Combined CNS	18	DFS (5 yr) = 11%	13	DFS (5 yr) = 15%	23	DFS (5 yr) = 41%
		Combined other	5	DFS (5 yr) = 0%	15	DFS (5 yr) = 40%	23	DFS (5 yr) = 50%
Lawson et al., 2000 *(174)*[a]	MRC UKALL R1 1991–95	Isolated	22	DFS (5 yr) = 5%	20	DFS (5 yr) = 35%	79	DFS (5 yr) = 51%
		Combined CNS	5	DFS (5 yr) = 0%	17	DFS (5 yr) = 41%	21	DFS (5 yr) = 81%
		Combined other	2	—	12	DFS (5 yr) = 25%	19	DFS (5 yr) = 58%

Abbreviations: EFS, event-free survival; DFS, disease-free survival; CNS, central nervous system; BM, bone marrow.
[a]Definitions of time to relapse: early, <24 mo; intermediate, 24–36 mo; late, >36 mo after initial diagnosis.
[b]Definitions of time to relapse: early, <18 mo; intermediate, 18–36 mo; late, >36 mo after initial diagnosis.

Table 5
Summary of Chemotherapy / Radiotherapy Regimens and Results

Author	*Protocol*	*Induction*	*Consolidation*	*Maintenance*	*Irradiation*	*No. of patients*	*Outcome*
A. Early bone marrow relapse							
Buchanan 1990 *(103)* and 2000 *(115)*	POG 8303 1982–87	P, V, D, A	VM26/ARA-C	VM26/ARA-C, V/CP ± P, V, D, A	Therapeutic: Testis, 26 Gy	297	CR = 258 (87%) DFS (8 yr) = 8%
Henze et al., 1994 *(105)*	ALL-REZ BFM 83/85/87[a]	P, V, A, MTX, ARA-C	Alternating R1/R2	TG, MTX	Therapeutic: Testis, 24 Gy CNS, 24 Gy	146	CR = 113 (77%) DFS (8 yr) = 9%
Giona et al., 1997 *(104)*	AIEOP R-87	P, IDA, ARA-C	Multidrug regimen, SCT			73	CR = 55 (75%)
B. Late bone marrow relapse							
Pui et al.,1988 *(106)*	St.Jude <1983	P, V, D, IT	Alternating MP/MTX and V/CP, P, V, D		Therapeutic: Testis, 24 Gy CNS, 24 Gy	26	CR = 26 (100%) EFS (5 yr) = 31%
Sadowitz et al., 1993 *(107)*	POG 8304 1983–89	P, V, D, TIT	MP/MTX, V/CP, P, V, P, D vs. VM26/ARA-C P, V, D vs.VM26/ARA-C, TIT		Therapeutic: Testis, 26 Gy CNS, 24 Gy	105	CR = 102 (97%), EFS (4 yr) = 37%
Henze 1994 *(105)*	ALL-REZ BFM 83/85/87a	Alternating R1 and R2		TG/MTX	Therapeutic: Testis, 24 Gy CNS, 24 Gy	183	CR = 172 (94%) DFS (8yr) = 39%
Rivera et al., 1996 *(35)*	St. Jude R11 1983–90	P,V,A,VM26, ARA-C, TIT	Alternating VP16/CP and MTX/MP VM26/ARA-C, P/V		Therapeutic: CNS, 24 Gy	34	CR = 33 (97%) EFS (5 yr) = 65%

Continued

Table 5 (Continued)
Summary of Chemotherapy / Radiotherapy Regimens and Results

Author	*Protocol*	*Induction*	*Consolidation*	*Maintenance*	*Irradiation*	*No. of patients*	*Outcome*
C. Isolated CNS relapse							
Winick et al., 1993 *(43)*	POG 8304 1983–90	P, V, D,TIT	MP/MTX, V/CP, VM26/ARA-C vs. P/D Late intensification: P, V, D vs. VM26/ARA-C, TIT		Early: Cranial, 24 Gy	120	EFS (5 yr) = 42%
Ribeiro et al., 1995 *(44)*	St. Jude R11 1983-89	P, V, A,VM26 ARA-C, TIT	V16/CP, MTX/MP, VM26/ARA-C, P, V, TIT		Late: Cranial, 24 Gy Spinal, 15 Gy	20	EFS (5 yr) = 70%
Henze 1997 *(1)*	ALL-REZ BFM 83/ 85/87/90	Alternating R1 and R2 (+ R3), TIT		TG, MTX, TIT Cranial,18/24 Gy	Late: ± Spinal,18/20 Gy	73	EFS (5 yr) = 42%
Gaynon et al., 1998 *(2)*	CCG 1983–89	Variable	Variable	Variable	Variable	220	DFS (6 yr) = 37%
Ritchey et al., 1999 *(17)*	POG 9061 1990-93	V, D, Dex, TIT	HD-ARA-C, A, HD-MTX,HD-MP, VP-16, CP, TIT	MP, MTX, V, CP	Late: Cranial, 24 Gy Spinal, 15 Gy + DEX, V, A	83	EFS (5 yr) = 70%
Lawson et al., 2000 *(174)*	MRC UKALL R1 1991–95	DEX, V, A, EPI, TIT	ARA-C, VP16, DEX, V, A, EPI, CP, TG, TIT	SCT or P, V, MP, MTX, ARA-C, VP-16, CP, TG, IT	Late: Cranial, 24Gy Spinal, 24 Gy	26	DFS (5yr) = 58%

Continued

Table 5 (Continued)
Summary of Chemotherapy / Radiotherapy Regimens and Results

Author	*Protocol*	*Induction*	*Consolidation*	*Maintenance*	*Irradiation*	*Diagnosis*	*No. of patients*	*Outcome*
D. Isolated testicular relapse								
Bleyer et al., 1986 *(129)*	CCG 1972–83	Variable	Variable	Variable	Variable	Overt	145	EFS (3 yr) = 40%
Nachman et al., 1990 *(19)*	CCG 160s 1978–83	P, V, A, TIT	MP/MTX, P,V		24 Gy bilateral	Occult	57	EFS (4 yr) = 65%
Uderzo et al., 1990 *(135)*	AIEOP REC80-ITR 1980–87	V, A, ARA-C, MTX, IT	Maintenance chemotherapy LSA2-L2		Early, 20 Gy bilateral	Overt	49	EFS (4 yr) = 41%
Buchanan et al., 1991 *(42)*	POG 8303 1983–87	P, V, D, A, TIT	VM26, ARA-C, TIT	Rotating VM26/ARA-C and V/CP ± P, V, D, A	Early, 26 Gy bilateral	Early overt	34	EFS (3 yr) = 44%
Wofford et al., 1992 *(21)*	POG 8304 1983–89	P, V, D,TIT	MP/MTX, V/CP, P,V, P, D vs. VM26/ARA-C, P, V, D vs.VM26/ARA-C, TIT		Early, 26 Gy bilateral	Occult Late overt	55 25	EFS (4 yr) = 53% EFS (4 yr) = 84%
Finklestein et al., 1994 *(175)*	CCG 112 1984–88	Modified BFM or New York protocol			24 Gy bilateral	Early overt	55	EFS (5 yr) = 43%
Grundy et al., 1997 *(134)*	UKALL 1972-87	Variable			24 Gy bilateral	Occult + overt	33	EFS (5 yr) = 59%
Wolfrom et al., (1997 *(15)*	ALL-REZ BFM 83-90 1983–95	Alternating R1 and R2,(R3), (T)IT		MTX, TG	Orchiectomy or 24 Gy (contralateral 12–24 Gy)	Overt	59	EFS (8 yr) = 53%
Gaynon et al., 1998 *(2)*	CCG 1983–89	Variable	Variable	Variable	Variable	Overt	112	DFS (6 yr) = 64%

Abbreviations: A, L-asparaginase; ARA-C, cytarabine; CNS, central nervous system; DFS, disease-free survival; CP, cyclophosphamide; CR, complete remission; D, daunorubicin; DEX, dexamethasone; EFS, event-free survival; EPI, epirubicin; HD, high dose; IDA, idarubicin; IFO, ifosfamide; IT, intrathecal methotrexate; MP, 6-mercaptopurine; MTX, methotrexate; P, prednisone; R1, PVA/MP/ARA-C/MTX/VM26/IT; R2, DEX/VDS/A/TG/MTX/IFO/D/IT; TG, 6-thioguanine; SCT, stem cell transplant; TIT, triple intrathecal therapy (MTX/P/ARA-C); V, vincristine; VDS, vindesine; VM26, teniposide; VP16, etoposide; POG, Pediatric Oncology Group; BFM, Berlin-Frankfurt-Münster Group; AIEOP, Italian Association for Pediatric Hematology and Oncology; CCG, Children's Cancer Group; MRC, Medial Research Council.

[a]ALL-REZ BFM 87 specified preventive cranial irradiation (12–18 Gy) in patients with bone marrow relapse.

supplemented by triple intrathecal therapy. The remission rate was 83%, increasing to 87% after a further 2-wk course of teniposide and cytarabine *(112)*.

With a more intensive reinduction treatment, as used by the BFM Relapse Study Group for children with early BM relapse, a remission rate of 77% could be achieved. The protocol consisted of a 4-wk schedule of prednisone, vincristine, L-asparaginase, intermediate- or high-dose MTX, and high-dose cytarabine, followed by two alternating 5–8-d multidrug courses, R1 and R2, containing glucocorticoids, thiopurines, Vinca alkaloids, epipodophyllotoxins, oxazaphosphorines, intermediate- or high-dose MTX, daunorubicin, cytarabine, and intrathecal MTX *(36,105)*. Failure to improve remission rates with a much more intensive treatment containing more drugs at higher cumulative doses might be partially explained by the more intensive frontline therapy of the BFM Group compared with that of the POG. It may be concluded that the remission rate in patient groups similar to these does not depend on the quantity and intensity of chemotherapy, suggesting a biologic limit to the efficacy of nonselective antiproliferative drugs. Other groups have published comparable results of reinduction therapy using different, but mostly less intensive regimens *(104)*.

In children with late BM relapse (i.e., later than 6 mo after cessation of frontline therapy), higher remission rates can be achieved. The largest patient series treated according to uniform protocols have been reported by the POG and the BFM Relapse Study Group. The reinduction schedule of the POG 8304 protocol consisted of a 4-wk course of prednisone, vincristine, and daunorubicin. The protocols ALL-REZ BFM 83–87 started reinduction treatment directly with the more intensive multiagent courses, R1 and R2. Again, remission rates were comparable: 97% in the POG series and 94% in the BFM series *(105,107)*. Similar results have been reported by other authors in smaller patient groups using reinduction regimens of variable intensity *(35,106)*. Patients with concomitant involvement of extramedullary sites have been included in the studies; they received local therapy (in general radiation therapy), to the involved anatomic site in addition to the systemic chemotherapy.

Until now, there has been no clear evidence that more intensive reinduction therapy for BM relapse of ALL leads to better remission rates. However, a recent retrospective analysis of the ALL-REZ BFM 90 trial indicated that dose intensity of the reinduction therapy might have influenced remission rates and outcome, as postulated by Hryniuk *(113)*. Patients with a short time interval between the first two chemotherapy courses had a significantly better remission rate and EFS, compared with results for patients with longer intervals *(114)*. This question is being prospectively addressed in the relapse trial ALL-REZ BFM 96, which aims to shorten the time intervals between the first courses of chemotherapy by randomized administration of granulocyte colony-stimulating factor and scheduled dose reductions, thereby increasing the dose intensity and improving the overall outcome.

Two general consideration are important when comparing remission rates and outcome between different trials. First, the rate of second CR may depend on the variable intensity of frontline protocols, so that a direct comparison of CR rates in relapse trials is difficult. Second, the remission status is conventionally assessed on the basis of morphology (i.e., an M1 marrow rating following reinduction therapy simply means that a 2-log reduction of the leukemic cell burden has been achieved). As we have recently learned from frontline trials, the rapidity and the extent of response are much more reliable predictors of long-term EFS than the mere morphologic assessment of remission. Whether prospective studies of minimal residual disease in relapsed ALL would provide better guidelines for the introduction of more intensive reinduction treatment, leading to a better quality of remission and eventually to a better long-term outcome, remains to be addressed.

3.1.2. Postremission Chemotherapy

A broad variety of drug combinations and schedules have been exploited as postremission chemotherapy (Table 5A,B). The POG performed postremission treatment of children with BM relapse on the basis of alternating drug pairs combined with reinduction courses. They consisted of weekly intermediate-dose cytarabine/teniposide alternating with weekly vincristine/cyclophosphamide, with or without standard reinduction courses for children with early BM relapse *(115)*. For children with late BM relapse, alternating pairs of standard maintenance dose methotrexate/6-mercaptopurine and vincristine/cyclophosphamide have been used, interrupted by reinduction pulses with prednisone/doxorubicin or, as a randomized alternative, with teniposide/cytarabine *(107)*. Both protocols contained extended triple intrathecal therapy. Cranial or gonadal irradiation (24 or 26 Gy) was applied in cases of concomitant leukemic involvement at different sites.

Postremission treatment in BFM relapse trials for patients with early or late BM relapse consisted of alternating multidrug courses, R1 and R2, to a total of eight. Standard-dose MTX/6-thioguanine was given for 2 yr as maintenance therapy *(36,105)*. Children with an isolated BM relapse received MTX intrathecally during the intensive multidrug courses only. In cases of concomitant CNS involvement, cranial irradiation was administered at the end of intensive treatment at a dose depending on the previously applied radiation therapy (maximum dose, 24 Gy), and triple intrathecal therapy was continued throughout the first year of therapy. Patients with gonadal involvement received local irradiation at a dose of 24 Gy, if the involved testis had not been surgically removed.

During the ALL-REZ BFM 87 trial, preventive cranial irradiation was introduced for all children with isolated bone marrow relapse because of an excess of subsequent CNS relapses in the preceding trial 85 and during the first period of trial 87. Retrospectively, children who received preventive cranial irradiation had a significantly better EFS rate than those not treated with irradiation *(116)*.

For patients with early BM relapse, long-term results have been disappointing. In both the POG and BFM Study Group, disease-free survival after 8 yr was <10% *(105,115)*. These unfavorable results are in agreement with other reports *(2,37,38)* but contrast with studies of children with late BM relapse, in which long-term survival rates range from 30 to 40% *(105–107)*.

Only a few randomized studies, analyzing the efficacy and toxicity of single drugs and drug combinations, have been per-

formed in children with relapsed ALL . Feig et al. *(117)* reported the 1996 results of a Children's Cancer Group (CCG) study in which patients were randomized to receive idarubicin (12.5 mg/m^2) vs daunorubicin (45 mg/m^2) during induction and consolidation therapy. Because of unacceptable toxicity, the dose of idarubicin was reduced to 10 mg/m^2 for the second half of the study. Whereas the EFS rate at 2 yr was significantly better for children who received the higher dose of idarubicin, this advantage was counterbalanced after 3 yr of follow-up by late adverse events in the idarubicin group *(117)*. A response rate of 50% with moderate toxicity has been reported for children with BM relapse who received idarubicin monotherapy at a dose of 24 mg/m^2 *(118)*.

The POG analyzed the efficacy of periodic 4-wk reinduction pulses given with vincristine, prednisone, daunorubicin, and L-asparaginase during maintenance therapy with alternating drug pairs (teniposide/cytarabine and cyclophosphamide/vincristine) in children with early BM relapse. They did not find any significant improvement in outcome compared with results for patients not receiving the reinduction pulses *(115)*. For children with late BM relapse, the POG analyzed the efficacy of teniposide (150 mg/m^2) and cytarabine (300 mg/m^2) twice a week vs daily prednisone (40 mg/m^2) and weekly vincristine (1 mg/m^2) besides other weekly rotating drug pairs. Event-free survival after 5 yr was not different between the two regimens *(107)*.

The BFM Relapse Study Group performed consecutive randomized trials to determine the optimal dose, infusion time, and folinic acid rescue schedule of high-dose MTX. In trial ALL-REZ BFM 85, MTX given at a dose of 12 g/m^2 as a 4-h infusion, followed by 12 doses of folinic acid (15 mg/m^2) every 6 h starting 24 h after the beginning of MTX infusion, was compared with MTX (1 g/m^2) as a 36-h infusion with only two doses of folinic acid administered at 48 and 54 h *(119)*. The randomization was prematurely stopped, as there was a clear trend toward an inferior outcome in patients given the higher dose of MTX. Patients in the intermediate-dose arm did slightly better; however, a higher toxicity was seen in the limb with MTX at 1 g/m^2 over 36 h, probably because of the longer exposure time *(36)*. In the ALL-REZ BFM 90 trial, the 36-h MTX treatment, followed by two doses of folinic acid (at 48 and 54 h), was randomly compared with MTX at 5 g/m^2 over 24 h with three doses of folinic acid (at 42, 48, and 54 h). No difference in EFS rates was seen between the two regimens *(120)*.

Despite various attempts to improve the prognosis for children with BM relapse of ALL, using intensive multiagent chemotherapy, it has not been possible to achieve a major breakthrough. Reported results are similar, regardless of the chemotherapy regimen used. Interestingly, the well-known prognostic factors identified at relapse of ALL, and probably also the intensity of the frontline protocol, are far more important than the details of the treatment design in determining clinical outcome after retreatment.

3.1.3. Treatment of CNS Relapse

Relapse in the CNS was a major obstacle to cure of ALL before the introduction of effective CNS protection *(121)*. With contemporary therapy, CNS relapses have become rare events; however, the management of CNS relapse remains one of the major challenges in pediatric oncology because of the treatment-related adverse long-term sequelae.

Approaches to the treatment of children with isolated CNS relapse, using local irradiation with or without moderately intensive systemic chemotherapy, resulted in poor survival rates *(122–124)*. Craniospinal irradiation in combination with mild systemic therapy yielded a better outcome than cranial irradiation *(125)*. In consecutive trials, the POG evaluated the efficacy of systemic and intrathecal chemotherapy as well as radiation therapy in children with isolated CNS relapse. In 1985, Land et al. *(126)* reported the results of a randomized comparison of early cranial irradiation (24 Gy) followed by extended triple intrathecal therapy vs early craniospinal irradiation (24 and 14 Gy), without subsequent extended triple intrathecal therapy, in combination with moderate systemic induction and maintenance chemotherapy. Only 5 (25%) of 20 patients relapsed after craniospinal irradiation, compared with 15 (55%) of 29 patients after cranial irradiation plus extended intrathecal therapy. The authors described a high rate of leukoencephalopathy after intrathecal and systemic chemotherapy following cranial irradiation and a excessive toxicity from systemic chemotherapy following spinal irradiation, leading to treatment delay in a number of patients *(126)*. In the subsequent POG trial 8304, 120 children with isolated CNS relapse received early cranial irradiation at 24 Gy after a standard induction regimen, followed by continuation therapy with rotating drug pairs and a late intensification. As part of the systemic treatment, the drug pairs teniposide/cytarabine and prednisone/doxorubicin were given in a randomized manner during continuation and late intensification therapy. Standard triple intrathecal therapy was applied weekly during induction therapy and monthly throughout the remaining treatment. The EFS rate was 42 ± 8% at 5 yr. The authors reported 35 (29%) subsequent BM relapses and 13 (11%) subsequent isolated CNS relapses. After adjustment for the most predictive factors, namely, initial remission duration and exposure to anthracyclines during frontline treatment, patients randomized to receive teniposide and cytarabine turned out to have a marginally yet significantly better EFS rate compared with the other group. The 17% rate of leukoencephalopathy associated with substantial acute and chronic neurotoxicity was remarkably high and led to discontinuation of therapy in some patients *(43)*.

Favorable results with delayed craniospinal irradiation allowing intensive systemic induction and consolidation chemotherapy have been reported by other groups, although the numbers of patients studied have been low *(44,127)*. Consequently, in the recently published trial POG 9061, irradiation was deferred for 6 mo to allow the delivery of maximally intensive systemic chemotherapy before craniospinal irradiation. During radiation therapy, systemic antileukemic treatment was administered with dexamethasone, vincristine, and thriceweekly L-asparaginase. Chemotherapy was substantially intensified over that in prior trials. After a standard induction regimen including dexamethasone instead of prednisone with weekly triple intrathecal therapy, patients received consolidation treatment that included two courses of high-dose cytarabine followed by L-asparaginase. Early intensification therapy consisted of four courses of intermediate-dose MTX (1 g/m^2 per

24 h), high-dose 6-mercaptopurine (1 g/m^2) as a 8-h infusion alternating with four courses of etoposide (300 mg/m^2) and cyclophosphamide (500 mg/m^2). During consolidation and intensification treatment, triple intrathecal therapy was given at monthly intervals for a total of five applications. Maintenance treatment after irradiation was of moderate intensity with standard 6-mercaptopurine/MTX alternating with vincristine/cyclophosphamide reinduction courses. Eighty-three patients were included in the study. The overall EFS rate was 70 ± 6% at 5 yr. For patients with a first remission duration of <18 mo, it was 46 ± 12%, compared with 81 ± 6% for patients with a remission duration of >18 mo. The rate of neurotoxicity, 6 (7%) of 83 patients, turned out to be remarkably reduced compared with results in prior trials and could be linked to intensive chemotherapy before radiation treatment in most cases *(17)*. In two other large published series of patients with isolated CNS relapse treated with a variety of regimens, the disease-free survival rate was 37 ± 3% (CCG; *n* = 220; *2*), or the EFS rate was 24–64% (UKALL; *n* = 98), depending on the duration of first remission *(38)*.

In the BFM relapse trials, patients with isolated extramedullary relapses have been uniformly treated with the same chemotherapy regimens used for systemic relapse, supplemented by local therapy. In one brief analysis including 73 patients with CNS relapse from several consecutive trials, the EFS rate was 42 ± 3% at 10 yr *(1)*. Chemotherapy consisted of the R1 and R2 courses, as for patients with BM relapse. However, the number of courses was restricted to four in the ALL-REZ BFM 83 trial and six in trials 85, 87, and 90. Furthermore, triple intrathecal therapy was intensified during the intensive treatment period and extended to 6 mo of maintenance therapy. Irradiation was always delayed until completion of intensive therapy and administered at doses adapted to the previously used dose and age. Cranial or craniospinal irradiation was employed according to preferences of the participating medical centers. In retrospective analyses, no significant difference in EFS rates could be found between the two radiation modalities. In patients treated according to the ALL-REZ BFM 83–96 trials, outcome according to time to relapse was similar to results reported by the UKALL group (Fig. 4). Interestingly, in a multivariate Cox regression model, male sex, T-cell immunophenotype, and older age at initial diagnosis proved to be independent adverse prognostic factors, whereas time to relapse lost prognostic significance after adjustment for these covariates *(128)*. The prognostic relevance of sex and age at initial diagnosis in children with isolated CNS relapse has been described by other study groups as well *(37,129)*.

Although recent results from the POG 9061 trial are superior compared with other data, no specific treatment elements can be determined to be responsible for the favorable outcome. Most CNS protective elements at even higher cumulative doses were included in regimens of other groups, such as the BFM, except for high-dose 6-mercaptopurine. One possible explanation for the good results of the POG trials might be the low number of patients with T-cell ALL: four (3%) in trial POG 8304 and three (4%) in trial POG 9061.

Table 5C displays treatment regimens and results of recently published representative trials. The appropriate treatment for children with isolated CNS relapse of ALL remains unclear and cannot be conclusively deduced from the published data. However, delay of irradiation to allow high-dose CNS effective chemotherapy seems to be a reasonable approach and is now employed in most trials. Postirradiation high-dose systemic chemotherapy should be avoided, and intrathecal therapy should be performed cautiously to prevent the fatal occurrence of leukoencephalopathy. Craniospinal irradiation may be advantageous compared with cranial irradiation, but this impression awaits confirmation by conclusive trials employing currently used high-dose systemic chemotherapy.

3.1.4. Treatment of Testicular Relapse

After BM and the CNS, the testicles are the third most frequent site of relapse. Isolated overt testicular relapses occur significantly later than isolated CNS relapses and comprise a group of relapses with a comparably favorable outcome *(13,46)*. Like any other relapse, an "isolated" testicular relapse has to be considered a systemic disease *(130–132)*. Even in the case of overt BM involvement, treatment results between isolated and combined relapses were not significantly different, suggesting that BM involvement is a consequence of reseeding by cells persisting in the testes that are still sensitive to chemotherapy *(15)*.

Table 5D summarizes the design and results of representative studies. In most reports, local therapy consisted of bilateral irradiation of the testes. The optimum dose of testicular irradiation is unclear. Most authors recommend bilateral testicular irradiation at doses above 22 Gy *(19,21,42,133,134)*, but persistent disease or subsequent local relapses have been reported after doses of 20–26 Gy in 5–7 % of the patients *(21,42,135)*. Severe gonadal dysfunction has been encountered after testicular irradiation at 24 Gy *(136,137)*, in particular if it is given to younger boys *(138)*. After doses of 12 or 15 Gy, Leydig cell function was sufficiently preserved to allow spontaneous pubertal development *(139)*.

For the reasons given above, the BFM Relapse Study Group recommends orchiectomy of a clinically involved (i.e., enlarged) testes and a biopsy of the contralateral testis. If the contralateral testis is histologically free of leukemia, radiation therapy should be given at a reduced dose of 15 Gy *(15)*. With this approach, local recurrences of only 2.5% (2/81) after isolated testicular relapse and 1.1% (1/87) after combined testicular relapse have been registered since 1983. This procedure seems to provide safe local control of the disease with the advantage of giving patients who have unilateral testicular relapse a chance to undergo spontaneous puberty. Furthermore, implantation of a testicular prosthesis may lead to cosmetically superior results compared with leaving atrophic testes without any hormonal function after 24 Gy of irradiation.

Time to relapse has again been found to be the most relevant prognostic factor in patients with isolated testicular relapse. Several attempts have been made to detect occult testicular leukemia by routinely performed open wedge biopsy of the testes upon completion of frontline therapy *(130,140)*. In 1990, Nachman et al. *(19)* reported a 10% rate of positive biopsy results. After standard reinduction therapy, concurrent bilateral testicular irradiation at 24 Gy, and standard maintenance therapy, a 4-yr EFS rate of 65 ± 14% was achieved. Of 16

patients with previously negative biopsies, yet suffering a late overt testicular relapse, 15 remained in continuous CR at 2 yr.

Occult testicular leukemia detected at completion of frontline treatment seems to represent resistant disease associated with an inferior outcome, in contrast to negative biopsies but late testicular recurrence of the leukemia. Treatment of patients with occult testicular leukemia according to POG protocol 8304 was less intensive than treatment of patients with an early overt testicular relapse according to protocol 8303. EFS rates in the two groups were comparable: 53% at 4 yr for patients with occult leukemia and 44% at 5 yr for patients with overt early testicular relapse *(21,42)*. Since no clear benefit could be demonstrated for routinely performed testicular biopsies at the end of frontline treatment, it has been abandoned in most protocols *(134)*. Other groups have confirmed the favorable results for boys with late isolated testicular relapse after intensive chemotherapy and local irradiation (EFS rate 66–76%) *(2,15)*.

The most adequate systemic therapy in addition to local treatment is difficult to determine because of the different patient populations included in reported studies, the different frontline protocols, and various criteria used for patient selection. High-dose MTX might be beneficial for patients with testicular relapse, since in children with primary ALL the incidence of testicular relapses could be significantly reduced by introducing intermediate- or high-dose MTX into frontline protocols *(136,137)*. In general, systemic therapy should probably be given as in patients with BM or CNS relapse.

3.2. Stem Cell Transplantation as Postremission Treatment

Although a second remission can be achieved in most of the children with relapsed ALL, its duration after chemotherapy alone is limited by subsequent relapse. SCT has therefore been introduced as substantially intensified postremission treatment. SCT affords the opportunity to administer chemo- and radiotherapy at doses that would be lethal without subsequent rescue of marrow function by BM or stem cell infusion. In addition, allogeneic SCT provides an antileukemic effect caused by a nonspecific reaction of donor immune cells against residual leukemic cells in the recipient. This graft-versus-leukemia (GvL) effect is thought to prevent subsequent relapses after allogeneic SCT, but it is also associated with graft-versus-host disease, a nonspecific reaction against cells of the recipient and a major reason for the higher treatment-related mortality associated with allogeneic SCT.

Thus, the most adequate treatment after remission induction in children with relapsed ALL remains a controversial issue. Whereas certain subgroups of patients can be cured with chemotherapy alone, others clearly benefit from SCT, which provides a better relapse-free survival rate despite its treatment-related mortality.

Comparison of the efficacy of SCT vs chemotherapy remains difficult. Most published data are based on retrospective analyses and are strongly biased by selection criteria, the effects of treatment at particular centers, gain of experience, and change of methods. Although patients with high-risk leukemia constitute the subgroup most often considered eligible for SCT, they must first achieve complete remission and be in a clinically stable condition before SCT is attempted (Table 6A,B).

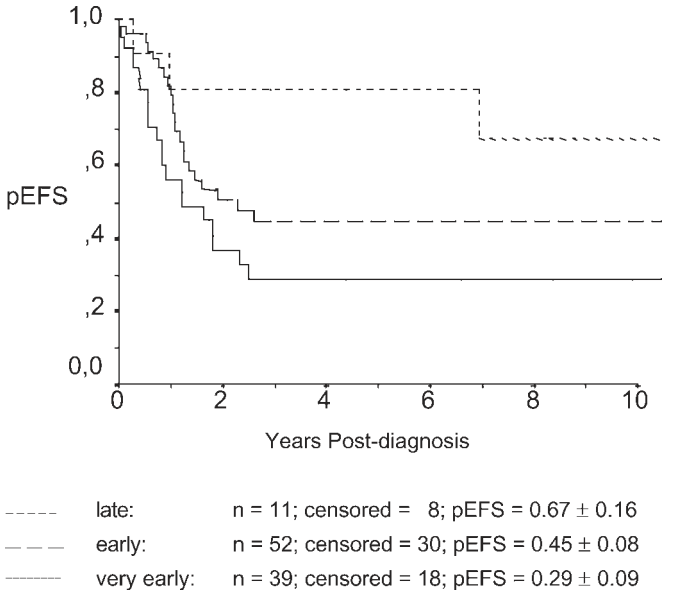

Fig. 4. Event-free survival probability (pEFS) for patients with isolated CNS relapse according to time of relapse (SCT censored; log-rank test, $p = 0.04$). Trials ALL-REZ BFM 83–95.

3.2.1. Allogeneic SCT from HLA-Matched Related Donors

Since the early 1970s, allogeneic SCT from HLA-matched related (mostly sibling) donors has been established as a treatment option for patients with leukemia in complete remission *(141,142)*. In most reports, total-body irradiation (TBI) combined with high-dose cyclophosphamide has been used as myeloablative conditioning regimen prior to SCT *(143)*, but a variety of other regimens, such as TBI/cytarabine *(144,145)*, TBI/etoposide *(146)*, or TBI and combinations of various cytostatic agents *(147)*, have been used as well. A retrospective analysis of the BFM Study Group suggests better results after TBI/etoposide compared with other regimens *(146)*.

There are no published studies with a prospective randomized comparison between allogeneic SCT and chemotherapy as postremission therapy. Several retrospective analyses have tried to overcome selection biases by stratification of patients according to risk factors and the interval from diagnosis to SCT or by matching pairs according to established risk parameters (Table 6A). In 1994, Barrett et al. *(148)* published treatment results for a large series of children with ALL in second CR after allogeneic SCT from matched related donors in the International Bone Marrow Transplant Registry, compared with results of chemotherapy alone according to POG protocols. The two patient groups were matched according to sex, age, immunophenotype, initial leukocyte count, and duration of first remission. The probability of EFS was significantly better for the SCT group, 40 ± 3% after SCT compared with 17 ± 3% for patients receiving chemotherapy only. The higher probability of treatment-related death after SCT, 27 ± 4% compared with 14 ± 4% after chemotherapy alone, was counterbalanced by a significantly lower probability of subsequent relapse, 45 ± 4% compared with 80 ± 3% after chemotherapy. Disease-free sur-

Table 6
Hematopoietic Stem Cell Transplantation Versus Chemotherapy

Author	*Protocol*	*Treatment*	*Statistical method*	*TRM (%)*	*Relapse rate (%)*	*Condtioning regimen*	*Type of relapse*	*No. of patients*	*Outcome (DFS)*
A. HLA-identical related donor									
Barrett et al., 1994 *(148)*	IBMTR 1983–91	RD-SCT	Matched-pair analysis	27	45	Variable	All	255	(5 yr) = 40%
	POG 1983–91	Chemotherapy		14	80	—	All	255	(5 yr) = 17%
Dopfer et al., 1991 *(146)*	ALL-REZ BFM	RD-SCT	Adjusted for time to SCT	18	20	TBI/VP16, others	All	51	(5 yr) = 52%
	1983–90	Chemotherapy				—	Late	165	(5 yr) = 41%
		Chemotherapy				—	Early	115	(5 yr) = 22%
Uderzo et al. 1995 *(149)*	AIEOP	RD-SCT	Adjusted for time to SCT	23	43	TBI/CP, others	All	57	(5 yr) = 41%
	1980–90	Chemotherapy		6	81	—	All	230	(5 yr) = 22%
Schroeder et al., 1999 *(150)*	Nordic countries	RD-SCT	Matched-pair analysis	21	29	Variable	All	75	(8 yr) = 40%
	1981–95	Chemotherapy				—	All	150	(8 yr) = 23%
B. HLA-matched unrelated donor or autologous SCT									
Wheeler et al., 1998 *(38)*	UKALL	RD/URD-SCT	Adjusted for time to	17	39	TBI/CP, others	All	110	(5 yr) = 40%
	1985–93	Chemotherapy	SCT and risk factors	5	65	—	All	261	(5 yr) = 26%
Oakhill et al., 1996 *(155)*	UKALL Bristol	URD-SCT	Single center	20	26	TBI/CP, CAMPATH-1	All	50	(2 yr) = 53%
Weisdorf et al., 1997 *(154)*	NMDP 1987–93	URD-SCT		48	17	Variable	All, adults included	106	(3 yr) = 42%
	UM, DFCI	Autologous SCT		14	76	Variable	All, adults included	98	(3 yr) = 20%
Ringden et al., 1997 *(157)*	EBMT, IMUST	URD-SCT	Matched-pair analysis			Variable	All, adults included	70	(2 yr) = 39%
	1987–94	Autologous SCT				Variable	All, adults included	140	(2 yr) = 31%
Borgmann et al., 1995 *(163)*	ALL-REZ BFM	Autologous SCT	Matched-pair analysis	4	65	Variable	All	52	(9 yr) = 26%
	1983–94	Chemotherapy		2	62	—	All	52	(9 yr) = 32%
Messina et al., 1998 *(161)*	AIEOP 1984–94	Autologous SCT		8	56	Variable	All	98	(8 y) = 34%

Abbreviations: CP, cyclophosphamide; DFCI, Dana-Farber Cancer Institute; DFS, disease-free survival; EBMT, European Group for Blood and Marrow Transplantation; IBMTR, International Bone Marrow Transplant Registry; IMUST, International Marrow Unrelated Search and Transplant Study; NMDP, National Marrow Donor Program; RD, related donor; SCT, stem cell transplantation; TBI, total-body irradiation; TRM, treatment-related mortality; UM, University of Minnesota; URD, unrelated donor. For other abbreviations, *see* Table 5 footnote.

vival after SCT was better than after chemotherapy, regardless of any initial risk factor.

The Italian Association for Pediatric Hematology and Oncology (AIEOP) Study Group *(149)* as well as the Nordic countries *(150)* compared results of patients after allogeneic SCT from matched related donors with those of patients after chemotherapy only. Whereas disease-free survival after SCT was significantly better for patients with early relapse, a significant difference could not be demonstrated in patients with late relapse or in those with extramedullary relapse. Similar findings have been reported by the UKALL group, indicating that patients with early marrow relapse (first remission duration, <2 yr) benefit significantly from allogeneic SCT (including 25% transplants from unrelated donors), whereas patients with later relapses do not *(38)*. The BFM Study Group has also reported that patients at high risk for subsequent relapse clearly benefit from matched related-donor SCT, whereas in patients with late marrow relapse (>6 mo after cessation of frontline therapy), the EFS rates after chemotherapy and SCT were comparable *(146)*. Consequently, the authors suggest that SCT in second CR may not be necessary for children with late relapse, even if a matched sibling is available, but that it could be preserved as a therapeutic option for patients experiencing a subsequent relapse and achieving a third CR *(151)*. A comparable approach is discussed by Barrett et al. *(148)*.

Recent as yet unpublished findings of the BFM Relapse Study Group have shown that the EFS rate for children with late marrow relapse is better after matched sibling-donor SCT than after chemotherapy, whereas overall survival is not different. A substantial proportion of children with a subsequent relapse after chemotherapy could be effectively salvaged even in third CR. In contrast, the outcome of patients with a relapse after allogeneic SCT was extremely poor, such that EFS and overall survival rates were not different.

In summary, SCT from HLA-matched related donors is an effective postremission therapy and should be performed in most patients with systemic relapse of ALL if a suitable donor is available, as is the case for 25–30% of the patients. It is associated with a higher treatment-related mortality and a lower relapse rate than chemotherapy alone. However, for subgroups including patients with an extramedullary relapse or late BM relapse, there is no clear benefit from SCT, and overall results appear to be similar or even better if SCT is preserved as an option for patients in third CR.

3.2.2. Allogeneic SCT from HLA-Matched Unrelated Donors

Since the late 1980s, BM donor registries have been established and provide the opportunity of SCT from unrelated donors for an increasing number of patients who lack a suitable related donor *(152)*. Unrelated-donor SCT carries an even higher risk for treatment-related mortality and morbidity, but it provides a better relapse-free survival rate than chemotherapy alone or allogeneic SCT from matched related donors *(153–156)*. Only a few reports on unrelated-donor SCT for childhood relapsed ALL are available (Table 6B), and in most instances authors refer to this method of transplantation as a treatment approach for various diseases with inhomogeneous patient cohorts *(152,153)*.

Results from a large series of patients registered at the National Marrow Donor Program (USA), on transplants from unrelated donors have been reported by Weisdorf et al. *(154)*. For patients younger than 18 yr of age, the disease-free survival rate in second CR was 47 ± 12% with a treatment-related mortality of 44 ± 12%. In 1997, Ringden et al. *(157)* reported data from the European Cooperative Group for Blood and Marrow Transplantation (EBMT) and the International Marrow Unrelated Search and Transplant (IMUST) study, including 70 patients who underwent unrelated-donor SCT in second CR. The patient cohort consisted of children and adults with a median age of 14 yr (range, 1–54 yr). The disease-free survival rate at 2 yr was 39 ± 6%. The authors did not find any prognostic significance for age in this setting.

Oakhill at al. *(155)* reported on a single-center study that included 50 patients with relapsed ALL in second CR. In most patients anti-CD52 monoclonal antibodies were used for ex vivo and in vivo T-cell depletion. Forty-two percent of the patients received allografts with one or more antigen mismatches, and 6% failed to engraft. The rate of treatment-related deaths was 20%, and the EFS rate at 2 yr was 53%. No difference in EFS could be demonstrated between patients with HLA-mismatched transplants and those with HLA-identical transplants *(155)*. The same group demonstrated the highly significant predictive value of minimal residual disease in the BM prior to SCT *(158)*.

No conclusive published comparative data on unrelated-donor SCT vs chemotherapy are available.

3.2.3. Autologous SCT

Rescue of marrow function with autologous BM or peripheral stem cells allows the administration of high-dose myeloablative radio- and/or chemotherapy, but it lacks the allogeneic GvL effect. Autologous SCT has been introduced for patients eligible for further intensification of postremission therapy without a suitable related donor. A variety of methods have been employed, including different preparative regimens and purging procedures to remove residual leukemic cells from the autograft. The design and results of recent trials are summarized in Table 6B, which compares autologous SCT with chemotherapy or allogeneic SCT.

In an early report by Kersey et al. *(159)*, a heterogeneous group of children and adults with high-risk ALL in first, second, or higher CR received either allogeneic SCT from related donors, or alternatively, autologous SCT after high-dose radio/chemotherapy. The EFS rates for these groups were similar *(159)*. Billett et al. *(160)* reported a disease-free survival rate of 53 ± 7% at 3 yr in children in first or subsequent ALL relapse and with a first remission duration >24 mo. The AIEOP found an EFS of 34 ± 5% at 8 yr in 98 patients receiving autografts in second CR after different conditioning regimens. Patients with an isolated extramedullary relapse had a significantly better EFS than did those with a BM relapse: 68% compared with 18% *(161)*. Furthermore, the disease-free survival rate for patients with isolated CNS relapses was significantly better after autologous SCT than after conventional chemo/radiotherapy, after adjustment of prognostic factors and time to SCT *(162)*. In contrast, the BFM Relapse Study Group reported a similar outcome after either autologous SCT or chemotherapy

in a patient group matched for the most relevant risk factors, namely, duration of first CR, site of relapse, immunophenotype, age, and gender. Results were similar regardless of the duration of first CR. However, 85% of the patients had an isolated or combined BM relapse *(163)*.

Results for a large series of patients have been published by Ringden et al. *(157)*, who compared the outcomes of patients receiving either autologous or matched sibling-donor SCT in second CR. A lower transplant-related mortality after autologous SCT was counterbalanced by a higher relapse rate, resulting in similar overall EFS rates with both methods *(157)*. This effect was even more evident in comparisons of autologous with unrelated-donor SCT. For patients younger than 18 yr, no significant difference in EFS after these approaches could be demonstrated *(154)*. However, including adults, the EFS rate after autologous SCT was found to be significantly worse compared with allogeneic SCT.

At present, autologous SCT does not appear to be more effective than intensive chemotherapy in patients with BM relapse, although some evidence exists that autologous SCT might have a higher efficacy than chemotherapy in patients with isolated extramedullary relapse, particularly CNS relapse. The most likely explanation is the lack of a GvL effect by autologous SCT, which possibly acts like maintenance therapy, a well-known essential component of treatment for ALL. Attempts have been made, therefore, to supplement autologous transplantation by immunologic and/or genetic therapeutic elements in order to mimic the GvL effect. However, until now, most transplantation centers prefer the allogeneic SCT because of its better antileukemic effect, despite higher transplant-related morbidity and mortality rates.

3.3. Experimental Approaches

Donors with variable degrees of HLA mismatch have been accepted for allogeneic SCT, if fully matched donors have not been available. The degree of HLA mismatch was associated with treatment failure in the setting of related- or unrelated-donor SCT *(164,165)*. SCT from related donors sharing only one haplotype with the recipient resulted in high treatment-related toxicity and mortality rates or (if the allograft was fully T-cell-depleted) in a high rate of rejections and subsequent relapses *(166)*.

The feasibility of SCT with cord blood as the stem cell source has been shown, with use of donors having different degrees of HLA mismatches *(167)*. Allogeneic SCT after nonmyeloablative conditioning regimens has been described in selected patient groups, without convincing evidence provided for its general clinical applicability *(168)*. Borgmann et al. *(169)* proposed the continuance of treatment after autologous SCT with immunomodulating regimens, reinduction chemotherapy, and maintenance therapy. Alternatively, vaccination with gene-manipulated autologous leukemic cells has been considered to induce an antileukemic T-cell reaction by analogy to the allogeneic GvL effect *(169)*. Other immunogenetic therapeutic approaches show promising preclinical results but await further confirmation in phase I and II studies *(170–172)*. Radio- or chemolabeled monoclonal antibodies directed against leukemia-associated antigens have been used for reduction of tumor burden or for elimination of residual disease *(172,173)*.

Prospective phase I/II studies have to be performed in patients with high-risk ALL relapse to evaluate the feasibility and efficacy of these experimental approaches in comparison with established methods.

4. CONCLUSIONS AND PERSPECTIVE

A variety of prognostic factors have been identified in patients with childhood ALL in relapse. With currently used polychemotherapy, roughly one-third of patients who relapse in first remission will be cured. The EFS rates of subgroups range from nearly 0% up to >70%, depending on the individual risk profile. For children with systemic relapse, allogeneic SCT is currently thought to be the treatment of choice, since it allows maximum intensification of therapy for patients in second CR. By comparison with chemo/radiotherapy only, SCT is associated with a higher treatment-related mortality but a lower relapse rate. For patients with early systemic relapse who have a poor outcome after chemo/radiotherapy alone, a clear benefit from SCT could be demonstrated. However, there are others with extramedullary or late combined BM relapses, for example, for whom the indication of allogeneic SCT is less clear. For patients with an EFS rate extending from about 30% to >50%, who represent a substantial proportion of the patients with relapsed ALL, the appropriate postremission therapy remains to be determined.

The history of SCT is still young. Although at present we know fairly well the acute toxicity of SCT, which is considerable, in particular with unrelated transplants, we probably know only part of the adverse long-term sequelae, so that the potential risks and benefits of SCT have to be balanced carefully. The available, mostly retrospective studies do not give a clear answer as to whether SCT or intensive chemotherapy would lead to better rates of EFS and overall survival and finally to a better quality of life. As long as there are no clear criteria for SCT in large groups of patients with an intermediate prognosis, prospective controlled studies will be needed to compare efficacy, toxicity, and long-term results of chemotherapy vs SCT.

In frontline ALL, it has been shown that monitoring of minimal residual disease is a valuable technology to detect early response to treatment and thus to discriminate among good-, intermediate-, and poor-prognosis patients. Prospective studies are needed to show whether in patients with relapsed ALL, early molecular remission may define a group of children who have a good prognosis and do not need SCT as postremission therapy. Interestingly, in a recent report, minimal residual disease at a level of $\geq 10^{-3}$ before allogeneic SCT proved to be highly predictive for subsequent relapse, even after unrelated-donor SCT *(158)*. Thus, minimal residual disease monitoring might be able to identify patients who have highly resistant disease that cannot be eliminated even by the most intensive treatment.

If routine monitoring of minimal residual disease is introduced into frontline therapy, and patients already in first CR with persistent leukemia by this assay are candidates for SCT, then the group of patients with relapsed ALL would be completely different in the future, insofar as the majority would

already have received transplants as part of their first treatment. Such a strategy is justified if indeed more patients could be cured by frontline therapy. For those who relapse despite SCT, the outcome of conventional salvage treatment would be clearly worse than now.

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CHEMOTHERAPEUTIC STRATEGIES

II

G

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

15 Treatment of B-Cell Acute Lymphoblastic Leukemia

Perspective 1

CATHERINE PATTE

CONTENTS

1. INTRODUCTION

Mature B-cell acute lymphocytic leukemia (B-ALL) was recognized in the mid-1970s as a rare subtype (2–4%) of childhood ALL cases *(1)*. This disease appeared to be more aggressive than other leukemias, often with lymphomatous tumors and poor response to treatment. Leukemic cells were characterized by L3 morphology according to the French–American–British (FAB) classification and by the expression of monoclonal surface immunoglobulin (SIg). A relationship between Burkitt's lymphoma and L3 leukemia was evoked early. It appeared that these diseases were in fact different forms of the same disease (B-cell disease) : the tumoral cells had the same cytologic and immunologic features and displayed the same specific nonrandom chromosomal translocations, t(8; 14)(q24; q32), t(2; 8)(p12; q24), and t(8; 22)(q24; q11). The disease is also characterized by a high proliferation rate and a short doubling time, by a great propensity to disseminate and invade organ systems, in particular the central nervous system (CNS), by a poor response to conventional therapy, and by very early relapses.

By definition, B-cell diseases with >25% blasts in bone marrow are called B-ALL. Thus B-ALLs include diseases arising from the bone marrow, as well as diseases arising from extramedullary sites such as the abdomen but with bone marrow invasion. B-ALLs are therefore clinically heterogeneous, with a broad spectrum of clinical manifestations, ranging from the typical leukemia presentation with symptoms related to bone marrow involvement and/or myelosuppression (<20% of cases), with or without lymph node and spleen enlargement, to a presentation evoking a typical lymphoma with diseaserevealed by a large tumor mass, generally abdominal, but with partial bone marrow involvement at initial workup. Even in cases with extensive marrow involvement, a high white blood cell count is unusual in blood samples, the median being around 10×10^9/L *(2,3)*. Extranodal lymphomatous tumors are frequent, involving not only the liver and kidneys, but also the abdomen (gut tumors and ascites), the ovaries, the Waldeyer ring and the maxillaries, and even the thorax, with pleural effusion and sometimes mediastinum enlargement. CNS involvement may be present at diagnosis in up to 35% of cases *(4)*. Disseminated B-cell non-Hodgkin's lymphoma (B-NHL) and B-ALL share a similar dismal prognosis. Efforts to improve the outcome of therapy for patients with B-NHL greatly benefited B-ALL patients as soon as they were included in the same therapeutic protocols. It is noteworthy that in recent publications, most patients with B-ALL are described together with those for stage IV NHL, making it difficult in some instances to segregate results pertinent only to B-ALL.

Among the more notable treatment results are those obtained in Europe by the German–Austrian group (BFM studies) and the French Pediatric Oncology Society (LMB studies) and in the United States by the St. Jude team in Memphis, as well as the Pediatric Oncology Group (POG) and the National Cancer Institute (NCI).

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2. HISTORICAL EVOLUTION OF TREATMENT STRATEGIES

At the end of the 1970s, it was recognized that Burkitt's or B-cell NHL had to be treated differently from lymphoblastic NHL, the first with short intensive treatment and the second with long-term leukemia-like regimen *(5,6)*. During the early 1980s, different protocols clearly improved the outcome of advanced-stage Burkitt's lymphoma, but bone marrow and CNS involvement remained factors predicting a worse outcome *(7,8)*. By the end of the 1980s, treatment strategies had become more intensified, and results were clearly improved for this group of high-risk patients, including those with L3 ALL *(3,4,9,10)*.

This part of the chapter traces the evolution of different group strategies for the treatment of B-cell malignancies, drawing attention to current therapeutic guidelines for B-ALL and exploring the remaining questions and controversies. Outcome data are summarized in Table 1 of Chapter 16, by Sandlund.

2.1. The LMB Studies

The French Pediatric Oncology Society (SFOP) began developing its LMB studies for B-cell malignancies in 1981. Very briefly, the LMB protocols specify a prephase with prednisone and a low dose of vincristine (VCR) and cyclophosphamide (COP course), followed by two consecutive and intensive induction courses, called COPADM, based on fractionated cyclophosphamide (CPM) and high-dose methotrexate (HD MTX) (3 g/m^2 in a 3-h infusion) in combination with Adriamycin (AD; doxorubicin), VCR, and prednisone. The following two consolidation courses are based on cytarabine administered as a continuous infusion over 5 d. Remissions are maintained with the same drugs used during induction and consolidation, with treatment duration varying by individual study.

Forty-six B-ALL patients were treated in the first two LMB studies, trial 81 and trial 84 *(11)*. Among the 34 patients without CNS involvement, 25 (73%) survived, 23 (68%) of whom were in first complete remission (CR), 1 in second CR, and 1 in CR after bone marrow transplantation (BMT) for partial response. Among the 12 patients with CNS involvement, only 1 survived. It is important to note that relapses occurred preferentially in the CNS among patients with >70% blasts in the bone marrow. These results were similar to those observed in patients with Burkitt's NHL, that is, results were improved for most cases of advanced stage disease, except those with CNS involvement.

The following LMB 86 and LMB 89 studies were designed with attention to results of a phase II study of high-dose cytarabine and etoposide (CYVE course), showing three CRs among four patients with CNS disease *(12)* and the results of a series of B-ALL cases (with >90% blasts in the bone marrow) treated at St. Louis Hospital, showing that CNS disease was the major problem, either at diagnosis or relapse, and that intensive intrathecal treatment was in part responsible for the cure of four of the eight patients with CNS involvement *(13)*.

The LMB 86 study was designed for patients with or at high risk for CNS disease (B-ALL with >70% blasts in bone marrow) *(10)*. CNS therapy was intensified with a higher dose of MTX (8 g/m^2), a consolidation phase with two CYVE courses, more intensive IT therapy, and the addition of cranial irradiation. Among the 27 patients with L3 ALL who were included in this study, 9 of 11 (82%) and 11 of 16 (69%) without and with CNS involvement, respectively have been cured.

In the LMB 89 study, patients with <70% blasts in the bone marrow without CNS involvement were treated in the standard-risk group (group B), similar to LMB 84, whereas those with >70% blasts in the bone marrow and/or with CNS involvement were treated in the high-risk group (group C), similar to LMB 86, but with cranial irradiation performed only in cases with CNS involvement *(4)*. One hundred patients with L3 ALL have been treated, 11 in group B and 89 in group C. Thirty-five had CNS involvement. The overall survival at 5-yr was 89%, with an event-free survival (EFS) rate of 88% (92 and 83%, respectively, for patients without and with CNS involvement).

In conclusion, the survival of patients with B-ALL has been greatly improved through the LMB studies, largely as the result of induction therapy based on fractionated CPM, HD MTX, intensive intrathecal (IT) therapy, and a consolidation phase based on high-dose and continuous-infusion cytarabine + etoposide. CNS involvement remains a factor predicting a worse outcome.

2.2. The BFM Studies

Coincident with the LMB studies, the German-Austrian group conducted consecutive studies that also advanced the cure rate for B-ALL patients, as reported by A. Reiter *(3,14)* In the Berlin-Frankfurt-Münster (BFM) protocols, after a cytoreductive phase with corticosteroids and CPM, treatment consists of eight and then six alternating 5-d courses. In BFM 81 and 83, fractionated CPM, intermediate-dose MTX (0.5 g/m^2 in a 24-h infusion), and IT MTX were administered in each course, with cytarabine/teniposide alternating with doxorubicin. Cranial irradiation was recommended for all patients. In study 83, intraventricular therapy was introduced for patients with CNS involvement. In these two studies, 46 patients (15 CNS+) were enrolled. The 5-yr EFS rates in studies 81 and 83 were 43% (11% SD), and 50% (10% SD), respectively, for all the patients, and 57% (13% SD) and 53% (12% SD) for those without CNS involvement *(3)*.

In the BFM 86 study, treatment was intensified by increasing the dose of MTX to 5 g/m^2, by introducing VCR and triple IT injections in each course, by replacing CPM with ifosfamide in every second course, and by fractionating the administration of cytarabine/teniposide. Cranial irradiation was omitted altogether. Forty-one patients, all CNS–, were enrolled. The 5-yr EFS rate increased significantly to 78% (6% SD). The failures were three toxic deaths and six tumor progressions, five in local (abdominal) sites. There was only one CNS relapse *(3)*.

In the recently reported BFM 90 study, the treatment strategy for B-ALL was identical to the previous one, except for the introduction of high-dose cytarabine/etoposide (CC course) for patients in partial remission after two courses. Fifty-six patients with B-ALL were treated, 14 of whom had CNS disease. The EFS rate was 74% (SE = 6%). Notably, among the 13 adverse events occurring within the first year, 7 were related to toxicity and 6 to tumor progression *(14)*.

In conclusion, the introduction of HD MTX was the main factor in the improved survival observed in the BFM 86 and 90 studies. CNS disease was satisfactorily controlled with the

introduction of intraventricular therapy in studies 83 and 90. The omission of the cranial irradiation did not seem deleterious for the prevention or treatment of CNS disease. One of the remaining problems was toxic deaths.

2.3. St. Jude and POG Studies

In 1981, at St. Jude Children's Research Hospital, developed an improved treatment for advanced B-cell malignancies, the Total Therapy B regimen *(7)*. It consisted of cycles of fractionated CPM, VCR, and doxorubicin alternating with HD MTX (1 g/m^2 in a 24-h infusion) and escalating doses of infusional cytarabine. The 2-yr EFS estimate was 81% for the 17 stage III patients, but only 2 of the 12 stage IV and B-ALL patients (8 CNS+) were cured.

With the aim of improving on these results and previous ones *(15)*, the POG modified the St. Jude regimen by substituting high-dose cytarabine (3 g/m^2 every 12 h × 4) for continuous infusions of this agent and by intensifying IT chemotherapy (POG 8617 study) *(9)*. Four (then three) cycles of therapy were to be delivered for approx 4–6 mo. Seventy-four B-ALL patients (19 CNS+) together with 59 stage IV patients (17 CNS+) were enrolled on this protocol. Eleven died from toxicity (metabolic complications and infections) and 15 from resistant tumor (the sites of failure were not detailed for the B-ALL patients). The 4-yr EFS rate was 65% ± 8% (SE). Thus, the introduction of high-dose cytarabine in addition to intensified IT treatment clearly improved the outcome for B-ALL patients. Toxicity represented 42% of the causes of death.

Although not directed to B-ALL, but to stage III B-NHL, the POG 8616 study should be mentioned *(16)*. Patients were randomized to receive either regimen A (CPM, HD MTX, VCR, prednisone, and IT MTX), or regimen B (Total Therapy B, which in addition to regimen A included doxorubicin in the induction phase and cytarabine in the infusion phase). Regimen B produced a higher remission rate and thereafter a higher EFS rate.

2.4. The UKCCSG Studies

For the treatment of stage IV and B-ALL patients, the United Kingdom Children's Cancer Study Group (UKCCSG) developed in 1985 an intensive 6-mo chemotherapy regimen called MACHO, which specified successive courses of fractionated CPM, VCR, doxorubicin, HD MTX (2.5 g/m^2 in a 21-h infusion), high-dose cytarabine (2 g/m^2 every 12 h × 6), and triple IT injections (given by intraventricular reservoir in patients with CNS disease) *(17)*. Eleven patients with B-ALL were included in the study, two of whom had CNS disease. Seven patients (64%) survived, three after high-dose chemotherapy and BMT.

In the following study, the United Kingdom investigators adopted the French LMB 89 group C regimen, which they called the UKCCSG 9003 protocol *(18)*. The recommended CNS irradiation was performed in only one-third of the patients with CNS involvement. Forty-four patients with B-ALL (16 CNS+) were treated between June 1990 and February 1996, together with 19 patients with stage IV disease (all were CNS+). The EFS rate was 69% (CI, 57–79%). Of the 19 adverse events, 7 (37%) were toxic deaths, and among the 10 patients who relapsed, treatment had to be modified or delayed for 6.

In conclusion, these intensive treatments based on fractionated CPM, HD MTX, high-dose cytarabine, and intensive IT (or intraventricular) therapy improved previous results, but toxicity remained a problem. Importantly, the main difference between the French and the British trials administrating the same regimen was the use of urate oxidase in France, which seemed to prevent many of the metabolic complications and subsequent intolerance of chemotherapy observed in the British series.

2.5. The NCI Study

In 1989, I. Magrath at the NCI developed an intensive treatment for advanced-stage Burkitt's lymphomas, including patients with >25% blasts in the bone marrow, reported as stage IV disease *(19,20)*. The protocol 89-C-41 consists of two alternating chemotherapy regimens: A [CPM, doxorubicin, VCR, HD MTX (6.7 g/m^2 in 24 h), and IT MTX, and cytarabine] and B (ifosfamide, etoposide, high-dose cytarabine, and IT MTX). Fourteen (78%) of the 18 patients with bone marrow involvement were cured. All the failures were due to resistant tumor.

2.6. Other Studies

Other national groups *(21,22)*, generally using one of the previously described protocols, or single centers *(23)*, have reported on small series of patients with B-cell ALL, often enrolling them on the same protocols used for B-cell NHL.

2.7. Treatment of Relapse

With recent protocols, relapses in B-ALL patients are rare but are very difficult to treat. In published series, most of the patients who relapsed died. Details on relapse sites and the treatment of patients alive after relapse are generally not given, except that they received high-dose chemotherapy with autologous or allogeneic BMT. The only two patients treated in the LMB 81, 84, and 89 studies who survived after relapse are patients with isolated CNS relapses who achieved CR after CNS-directed therapy, followed by high-dose chemotherapy with autologous BMT.

The first difficulty is to find an effective second-line treatment, as all useful drugs have been used during primary treatment. The only report found on new drugs for relapsed B-ALL concerns topotecan, which induced a CR and a partial remission in two patients who had relapsed after high-dose chemotherapy with BMT.

Once a second remission is induced, high-dose chemotherapy with BMT is indicated, although the superiority of allogeneic vs autologous BMT remains to be demonstrated.

2.8. Problem of Atypical B-ALL

The vast majority of B-ALLs have the cytologic, immunologic, and cytogenetic characteristics described in the Introduction. However, some cases present with discordant features *(24,25)*, raising questions as to their most efficacious treatment. Most investigators recommend that cases of L3 morphology (without SIg positivity) should be treated on B-ALL protocols, as they have a translocation involving the *myc* oncogene. By contrast, cases with L1/L2 morphology (but with SIg positivity) should be treated on protocols for B-cell precursor disease, unless they present with a cytogenetic abnormality involving the myc locus. For other discordant cases, treatment is a matter of individual decision.

3. GENERAL GUIDELINES AND CONTROVERSIES

During the last 15 yr, major improvement has been achieved in the treatment of B-ALL, with cure rates now ranging from 65 to 90%, even when the CNS is involved. The biologic characteristics of disease and analysis of the previously reported series allow the following conclusions and questions.

1. Burkitt's lymphoma and leukemia are characterized by very high growth fractions (approaching 100% in most cases) and very short doubling times. Thus treatment must involve *intensive short multiagent chemotherapy* given in 3–5 d courses with a schedule characterized by fractionation and continuous infusion of drugs. The purpose is to maintain cytotoxic serum drug concentration over at least 48–72 h, a period during which every malignant cell should have a chance to enter the cell cycle.
2. Also, because of the rapid tumor cell doubling time and the potential for tumor regrowth before bone marrow recovery, the courses have to be administrated with the *shortest possible intervals* in between. Treatment delays due to toxicity in the UKCCSG 9003 trial might have compromised the end results.
3. Burkitt's lymphoma and leukemia are sensitive to many drugs. The single-agent phase II studies were mainly done in African Burkitt's tumors *(26)*, but nonendemic disease seems to have a similar response to chemotherapeutic agents. In B-ALL, *high doses* of some of these effective drugs are necessary to obtain clinically significant responses.

   From a review of the most successful studies, it can be said that the *three major drugs* are cyclophosphamide, HD MTX, and cytarabine.

   a. *Cyclophosphamide* is a classic drug with long-standing efficacy against Burkitt's tumor. It should be given on a fractionated schedule, on 3–5 consecutive days, or every 12 h for six doses, at a minimum dose of 1 g/m^2 per course.
   b. *Methotrexate* had also shown its efficacy, when given at low doses as a single agent *(27)* or in protocols such as COM or COMP, and at high doses as a single agent *(28)*. HD MTX is a necessary agent in the treatment of B-ALL, as clearly demonstrated by BFM trials and other successful studies. However, many questions remain about HD MTX: Which dose should be used within the range 1–8 g/m^2 specified by contemporary protocols? Are short infusions (3 or 4 h in the LMB studies) as effective as long infusions (24 h as in most protocols)? This last point is being investigated in the ongoing BFM 95 study.
   c. *Cytarabine* must be given either in repeated doses, in continuous infusions or in high doses. The LMB 86 and 89 studies and a POG study demonstrated the superiority of high vs low doses even in continuous infusions.
   d. *Vincristine* demonstrated its efficacy against Burkitt's tumors in an early phase II study, producing a response rate of 81% and a CR rate of 48% *(29)*. Hence, this drug has a place in multiagent polychemotherapy regimens for B-ALL.
   e. *Doxorubicin* was not tested in phase II studies of Burkitt's tumors, but it is used in all successful protocols. This drug may have contributed to the superiority of regimen B over regimen A in the POG 8616 study; however, a randomized Children's Cancer Group (CCG) study did not show any benefit of adding daunomycin to the COMP regimen for nonlympho-blastic lymphoma *(30)*.
   f. The *epipodophyllotoxins* are known to be effective against NHL/ALL in general, but the single-agent phase II studies were done at a time when these proliferations were not as well characterized as now. Teniposide and etoposide are combined with cytarabine or ifosfamide in most contemporary protocols (LMB 86, 89, BFM, NCI).
   g. *Corticosteroids* are used in many protocols. But apart from empirical clinical observations, there are no clear demonstrations of the sensitivity of B-ALL to corticosteroids. If corticosteroids are used, should dexamethasone be preferred to prednisone?
   h. *Ifosfamide* has been introduced in some protocols. In phase II studies, it has shown appreciable activity when combined with other drugs, especially etoposide *(31)* However, it was not demonstrated that it has equivalent or greater efficacy than cyclophosphamide or that their activities are non-overlapping. The POG is investigating in a randomized study the benefit of ifosfamide/etoposide in B-cell advanced-stage NHL and ALL.
4. Intensive *CNS-directed therapy* with preventive or curative intent is necessary in any protocol for B-ALL. HD MTX and high-dose cytarabine, besides their clear systemic effect, are essential components of such therapy because of their passage into the CNS *(32,33)*. Intensive local therapy also seems necessary, especially in the case of overt CNS disease. Is intraventricular therapy, which was successful in the BFM 83 and 90 studies, and for some patients in the MACHO and the POG 8616 studies, superior to IT therapy? This question may prove difficult to answer, as the insertion of an Omaya reservoir can be difficult to manage.

   It is now agreed that cranial irradiation is not necessary if the CNS is not involved. Its omission in several protocols, especially of the NCI and the BFM, did not affect clinical outcome, suggesting that cranial irradiation may be omitted when the CNS is involved.
5. The time to relapse has not changed with the intensity of treatment . Relapses still occur *early*, within the first year of treatment. This provides a supplementary argument for not prolonging therapy beyond 6–8 mo in B-ALL, contrary to strategies for other forms of ALL.
6. More than in any other type of ALL, *metabolic complications* are a major problem in the initial management of B-ALL. Because of the short doubling times of clonal proliferations, the resultant tumor masses are often huge, so that tumor lysis syndrome poses a serious hazard and must be carefully managed. A more generalized use of urate oxidase in the future should prevent some of the deaths observed in countries where this agent is not yet available. Urate oxidase might also prevent the loss of renal function, which is essential for good elimination of HD MTX and therefore timely administration of the planned therapy *(34,35)*. The prephase treatment in the LMB protocols allows one to manage the tumor lysis syndrome without

competing complications from other intensively administered agents and was not found to have an adverse impact on survival. It is interesting to note that, in the BFM 90 study, decreasing the duration of administration of CPM in the cytoreductive phase from 200 mg/m^2/d over 5 d to 2 d reduced the number of early deaths.

7. *Toxicity-related deaths* are another cause of failure. It was disappointing that randomized studies of granulocyte (macrophage) colony-stimulating factor did not demonstrate an advantage of these growth factors *(20,36)*. Nonetheless, treatment must be intensive to be successful, underscoring the need to manage patients in specialized centers, where clinicians must acquire experience with a given protocol, and to improve supportive care still further.
8. Because of the good results now achieved with conventional therapy, there is no *indication* for high-dose chemotherapy with hematopoietic stem cell transplantation in first CR.
9. *Relapses* occurring after treatment on a successful protocol have a very poor outcome. Generally, patients are resistant to all forms of salvage treatment. New drugs and new therapeutic approaches are urgently needed for these difficult situations.
10. If a second CR has been induced, *high-dose chemotherapy* with hematopoietic stem cell grafting has to be considered, although the preferred regimen is unclear. One possibility is BACT *(37,38)* or BEAM *(39)* with autologous BMT, which proved effective in relapsed Burkitt's lymphoma; another is high-dose busulfan *(14,40)*, together with high doses of other alkylating drugs. In B-ALL, allogeneic grafting would be preferred, at least in disease that primarily involves the bone marrow. So far, there has been no clear demonstration of a graft-versus-tumor reaction in B-cell disease. Autografting can be considered in cases with a lymphoma-like presentation that relapse in sites other than bone marrow.

4. CONCLUSIONS

B-cell ALL is now a curable disease in most cases. Although some therapy-related questions remain, the general guidelines for initial treatment are now well established and differ completely from those directing the management of other leukemias. Even so, current regimens are difficult to administer because of their related toxicity. A remaining challenge is to identify and assess new therapeutic strategies for relapsed B-ALL.

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16 Treatment of B-Cell Acute Lymphoblastic Leukemia

Perspective 2

JOHN T. SANDLUND

CONTENTS

1. INTRODUCTION

B-cell acute lymphoblastic leukemia (B-ALL) and Burkitt's lymphoma are generally discussed together because the distinction between the two is somewhat arbitrary. Children with an abdominal Burkitt's tumor and >25% marrow blasts are considered to have B-ALL, whereas those with <25% blasts are considered to have advanced-stage Burkitt's lymphoma with marrow involvement. Cytologically, the lymphoblasts in Burkitt lymphoma are characterized by a high nuclear-to-cytoplasmic ratio, prominent nucleoli, and vacuolated basophilic cytoplasm—referred to as L3 morphology according to the French–American–British (FAB) classification system *(1)*. Histologic examination of a Burkitt's tumor reveals sheets of monomorphic lymphoid cells with an associated "starry sky" appearance that results from the presence of interspersed tingible-body macrophages. Burkitt's lymphoma is an aggressive, high-grade malignancy, according to the National Cancer Institute (NCI) Working Formulation *(2)*. In the revised European–American lymphoma (REAL) classification system, these tumors are subcategorized into Burkitt's and "Burkitt-like" tumors *(3)*. The latter group is characterized by greater heterogeneity in cell size (i.e., a greater proportion of larger cells) and by the presence of a single large nucleoli rather than the multiple nucleoli typically observed in Burkitt's tumors. These two subtypes of Burkitt's lymphoma have not been reported to have any associated clinically significant differences; therefore, in the remainder of this discussion, Burkitt's lymphoma will be used to refer to all the small noncleaved cell lymphomas.

These tumors are of a mature B-cell immunophenotype, expressing surface immunoglobulin (usually IgM, although IgA or IgE can be detected in some cases) and various other B-cell markers (CD19, CD20, and CD21). They are characterized by one of three reciprocal chromosomal translocations involving the *MYC* protooncogene on chromosome 8 [t(8;14)(q24;q32), t(2;8)(p11;q24), and t(8;22)(q24;q11)] and one of the immunoglobulin genes *(3–6)*. These translocations result in the juxtaposition of the *MYC* protooncogene on chromosome 8 with one of the immunoglobulin genes, resulting in dysregulation of *MYC (6)*.

2. STATE OF THE ART TREATMENT

There have been striking advances in the management of Burkitt's lymphoma and B-ALL over the past 25 yr (Table 1) *(7–20)*. What was once a disease with a dismal outcome has become a curable entity. A randomized trial performed by the Children's Cancer Group (CCG), comparing two of the earliest successful antilymphoma treatment strategies (COMP and LSA2L2), demonstrated that for children with advanced-stage Burkitt's lymphoma, the cyclophosphamide-based COMP regimen was more effective than the multiagent LSA2L2 regimen *(13)*. These results formed the foundation for subsequent trials testing intensive alkylator-based strategies. Initial improvements over that achieved with COMP were achieved by the addition of high-dose methotrexate and in some cases high-dose cytarabine, even when the duration of therapy was shortened to 2–4 mo.

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Table 1
Treatment Outcome for Advanced-Stage (III, IV) Burkitt's Lymphoma and B-Cell ALL

Protocol [a]	*Stage*	*No. of patients*	*Event-free survival*		*Reference*
			Time (yr)	*Estimate (%)* [b]	
LMB 84[c]	BL-III	167	2	80 (SE 3)	*8*
	BL-IV+B-ALL (CNS–)	34	2	68 (SE 8)	
LMB 86[c]	B-ALL (CNS–)	11	>1	82 (SD 12)	*9*
	B-ALL (CNS+)	24	>1	75 (SD 9)	
LMB 89[c]	BL-III	278	5	91 (CI, 87–94%)	*10*
	BL-IV	62	5	87 (CI, 77–93%)	
	B-ALL	102	5	87 (CI, 79–92%)	
BFM					
81	B-ALL	22	5	40 (SD 6)	*11*
83	B-ALL	24	5	50 (SE 10)	*11*
86	B-ALL	41	5	78 (SD 6)	*11*
90	BL-III	169	6	88 ±3	*12*
	BL-IV	24	6	73 ± 10	
	B-ALL	56	6	74 ± 6	
CCG[c]					
LSA2L2 vs.	BL-III/IV+B-ALL	44	5	29 (CI, 16–43)	*13*
COMP	BL-III/IV/B-ALL	93	5	50 (CI, 39–60)	
COMP vs. D-comp	BL-III/IV/B-ALL	175	2	65	*14*
Orange vs.	BL-III/IV+B-ALL	43	1	83	*15*
LMB-86	BL-III/IV/B-ALL	42	1	84	
NCI[c]					
77-04	BL-III	30	3	57 ± 9	*16*
	BL-IV	9	3	13 ± 12	
CODOX/VIPA	BL-III/IV+B-ALL	75	1	89	*17*
BOSTON					
HiC-COM	BL-III	12	2	95 (CI, 54–99)	*18*
	BL-IV/B-ALL	8	2	50 (CI, 15–78)	
St. Jude Total B	BL-III	17	2	81	*19*
	BL-IV/B-ALL	48	2	17	
POG 8617	BL-IV	34	4	79 ± 9	*20*
	B-ALL	47	4	65 ± 8	

Abbreviations: BL, Burkitt's lymphoma; SE, standard error; SD, standard deviation; CI, 95% confidence interval. Abbreviations of cooperative groups and treatment regimens are given in the text.

[a] Treatment regimens are described in either the text or in appropriate references.

[b] Standard deviation or standard error indicated when specified in original sources.

[c] These studies include patients with B-lineage cell non-Hodgkin's lynphoma.

Further improvements in treatment outcome have been achieved over the past decade, mainly through further intensification of therapy (e.g., dose escalation of methotrexate and cytarabine) and the addition of new agents such as ifosfamide and etoposide. One of the most successful approaches has been the LMB-89 regimen designed by Patte et al. *(10)* of the French Pediatric Oncology Society (SFOP; Tables 1 and 2). With this regimen, children with <70% marrow blasts receive high-dose methotrexate (3 g/m^2), and those with >70% marrow blasts or central nevous system (CNS) disease receive methotrexate at 8 g/m^2 as well as courses of CYVE, which comprise higher-dose cytarabine and etoposide.

Equally successful results have been achieved by the Berlin–Frankfurt–Munster (BFM) group (Fig. 1). According to their recently published BFM-90 strategy, children with advanced-stage disease receive high-dose methotrexate at 5 g/m^2 *(12)*. Two additional ways in which BFM-90 differs from LMB-89 are in its use of both ifosfamide and cyclophosphamide, and in the use of high-dose cytarabine only for an incomplete response in the R2 risk group. Advances in treatment outcome have also been reported by the NCI, Pediatric Oncology Group (POG), and CCG. In sequential NCI studies, an improved outcome was achieved by the addition of ifosfamide, etoposide, and cytarabine (IVAC) to a regimen

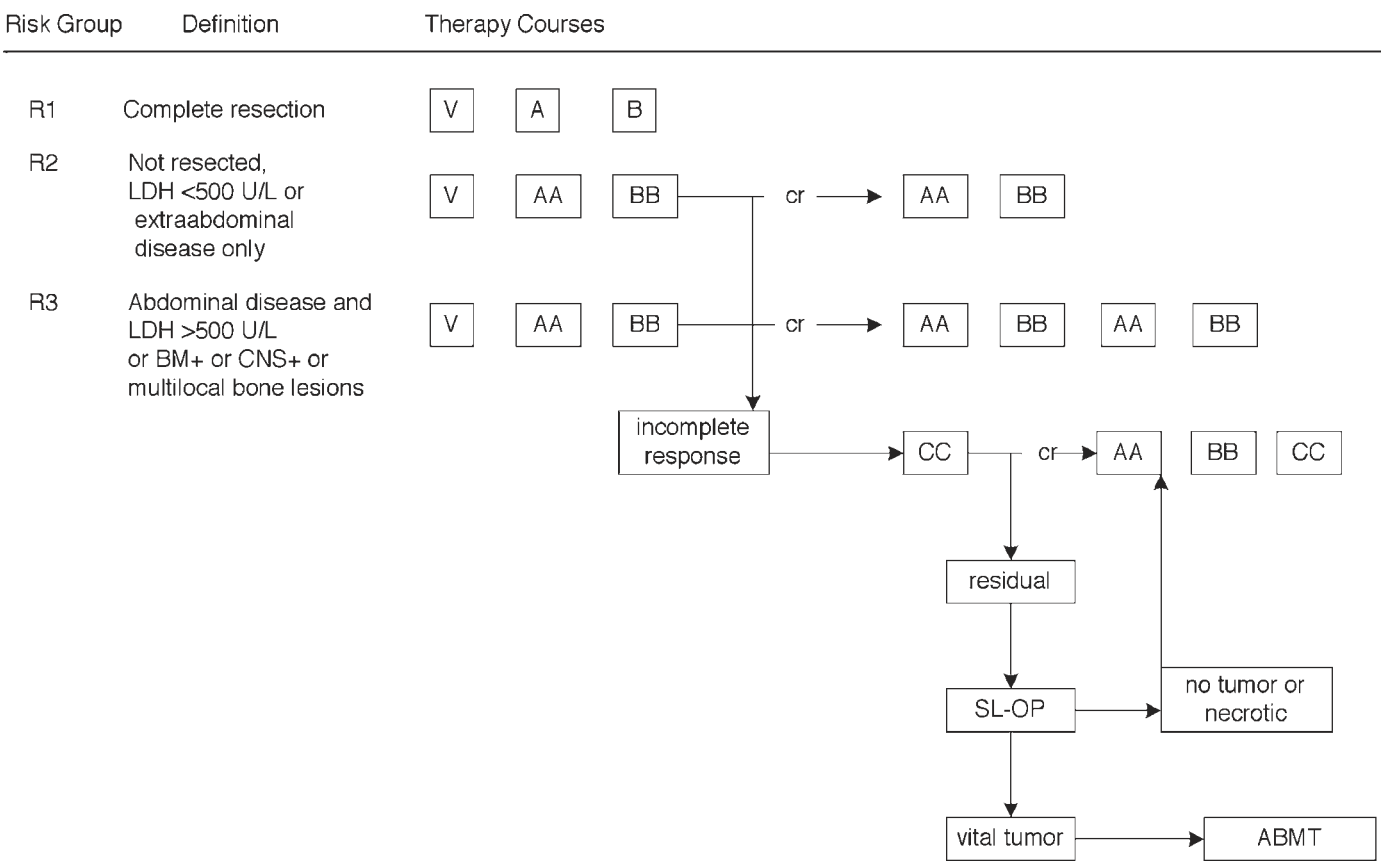

Fig. 1. BFM treatment strategy for advanced-stage B-cell ALL. Patients were stratified into three risk groups: R1, R2, and R3. V, cytoreductive prophase; cr, complete response; SL-OP, second-look operation; BM, bone marrow; ABMT, autologous bone marrow transplantation or blood stem cell transplantation; LDH, lactate dehydrogenase; CNS, central nervous system. Other treatment components are described in refs. *11* and *12*.

containing cyclophosphamide, vincristine, doxorubicin, and high-dose methotrexate (CODOX-M) *(17)*. POG investigators reported excellent results with a regimen featuring high-dose methotrexate and high-dose cytarabine *(20)*. Their current study is examining the value of an intensification phase emphasizing etoposide and ifosfamide. The CCG reported excellent results with the multiagent Orange regimen, which in a randomized trial appeared to have reduced toxicity compared with the LMB-86 regimen *(15)*.

3. ISSUES IN CLINICAL MANAGEMENT

3.1. What Are the Most Important Components of Modern Successful Therapy?

The most successful current treatments for B-ALL are generally quite similar in terms of both the overall strategy and the individual drug components. Even so, the primary components and differences in their applications are worthy of discussion.

3.1.1. Cyclophosphamide

Subsequent to the CCG trial of COMP versus LSA2L2, which demonstrated the superiority of the cyclophosphamide-based COMP regimen for the treatment of Burkitt's lymphoma, cyclophosphamide has remained the cornerstone of successful treatment regimens for Burkitt's tumor and B-ALL *(13)*. There are, however, some differences with respect to dose and schedule. In the LMB-89 regimen, during the first block of COPAD-M, patients receive 500 mg/m^2 daily for 3 d divided into q 12 h dosing fractions *(10)*. In COPAD-M #2, the dose of cyclophosphamide is doubled to 1 g/m^2 per d for a total of 3 g/m^2/course. By contrast, course BB in BFM-90 includes a lower dose of cyclophosphamide (200 mg/m^2/day for 5 d for a total of 1 g/m^2/course) given every 24 h rather than every 12 h *(12)*. Regimen A of the NCI CODOX-M/IVAC protocol includes cyclophosphamide at a dose of 800 mg/m^2 on d 1 followed by 200 mg/m^2 on d 2–5 for a total of 1.6 g/m^2 *(17)*. Among these regimens, there is no clear advantage of one approach over the other with respect to either total dose or schedule of fractionation (i.e., q 12 h vs q 24 h), in the context of the entire treatment plan. It does appear that cyclophosphamide should be given at a dose of at least 1 g/m^2/treatment block.

3.1.2. Methotrexate

Every successful B-ALL regimen incorporates high-dose methotrexate; however, the optimal dose and infusion duration remain to be established. In the LMB-89 regimen, group B patients receive 3 g/m^2 iv over 3 h, whereas group C patients receive 8 g/m^2 intravenously over 4 h *(10)*. In the BFM-90 regimen, courses AA and BB include methotrexate at a dose of 5 g/m^2 given iv over 24 h *(12)*. In regimen A of the NCI proto-

Table 2
Outline of LMB-89 Regimen of the French Pediatric Oncology Society[a]

Group	*Definition*	*Prephase*	*Induction x 2*	*Consolidation x 2*	*Continuation*
A	Resected stage I and resected abdominal stage II	NA	COPAD	NA	NA
B	Other stage II, III, IV or B-ALL with BM blasts <70%; no CNS involvement	COP	COPAD-M_3	CYM	1
C	B-ALL with >70% BM blasts; CNS involvement, group B COP failures (i.e., <20% reduction)	COP	COPAD-M_8	CYVE	1, 2, 3, 4

Abbreviations: BM, bone marrow; CNS, central nevous system; NA, not applicable. Drug abbreviations are given in the text.
[a]For further details, see ref. *(10)*.

col, a 1-h infusion of methotrexate at a dose of 1200 mg/m^2 is followed by a 23-h infusion at a dose of 240 mg/m^2/h for a total dose of 6.72 g/m^2 *(17)*. There is no clear advantage to any of the above approaches in the context of the overall treatment program given. Common to all, however, are the higher doses of methotrexate compared with the less intensive and less effective regimens in which methotrexate was administered at approximately 1 g/m^2.

3.1.3. Cytarabine

Cytarabine is used in most modern B-ALL regimens, but the optimal dosing strategy is controversial. In the BFM-90 regimen, low-dose cytarabine (150 mg/m^2 is given q 12 h on d 4 and 5 of block AA) *(12)*. In the LMB-89 regimen, group B patients receive low-dose cytarabine at a dose of 100 mg/m^2/d as a continuous infusion on d 2–6 (CYM block), whereas those in group C receive high-dose cytarabine at a dose of 3 g/m^2 in iv over 3 h on d 2–5 coupled with a low-dose 12-h infusion of 50 mg/m^2/d on d 1–5 (CYVE block) *(10)*. A high-dose strategy is also used in the NCI CODOX-M/IVAC regimen; in regimen B, cytarabine is given every 12 h on d 1 and 2 at a dose of 2 g/m^2 *(17)*. The results from these studies suggest that various dosing schedules for cytarabine may be acceptable depending on the context of the total package of therapy; an optimal schedule has not yet emerged.

3.1.4. Ifosfamide

The role of ifosfamide in the management of B-ALL is clearly more controversial than the role of cyclophosphamide, high-dose methotrexate, or cytarabine. Ifosfamide possesses activity as a single agent or in combination with other agents in patients with recurrent lymphoma who were heavily pretreated with cyclophosphamide *(21–28)*; however, there are no conclusive data comparing the relative effectiveness of cyclophosphamide vs ifosfamide in the treatment of B-ALL. Among current B-ALL regimens, the use of ifosfamide is variable. Although it is not used at all in the very successful LMB-89 regimen *(10)*, it is used in both the BFM-90 *(12)* regimen and the NCI CODOX-M/IVAC regimen *(17)*. Without a randomized trial, it is unlikely that this controversy will ever be resolved.

3.1.5. Etoposide

The LMB-89 *(10)*, BFM-90 *(12)*, and CODOX-M/IVAC *(17)* protocols all employ etoposide; an ongoing POG trial is determining the benefit of an ifosfamide/etoposide intensification phase. This drug was added to these regimens in an attempt to improve treatment outcome, but it is difficult to know the extent to which this agent contributes to overall outcome. Given the acceptable rate of secondary acute myeloid leukemia (AML) cases associated with these etoposide-containing regimens, coupled with the excellent overall cure rates being achieved, most investigators would be inclined to continue using this agent as currently dosed and scheduled.

3.2. What Is the Optimal Approach for CNS-Directed Therapy?

CNS-directed therapy for those who either have CNS disease at diagnosis [i.e., blasts in the cerebrospinal fluid (CSF) or cranial nerve palsies on physical examination] or are at significantly increased risk for developing it, uniformly includes high-dose systemic therapy (i.e., high-dose methotrexate with or without high-dose cytarabine), coupled with direct instillation of chemotherapy into the CSF. The route of administration of chemotherapy into the CSF is usually intrathecal through a lumbar puncture; however, an intraventricular route has been used by others, such as the BFM study group, for patients with overt CNS disease at diagnosis. With the BFM-90 regimen, a 6-yr event-free survival (EFS) rate of approx 65% was reported for patients who were CNS-positive at diagnosis *(12)*. Among CNS-positive patients treated on the LMB-89 protocol, the probability of long-term EFS was 79%. In contrast to the BFM approach, the LMB-89 regimen does not include intraventricular drug delivery. A current St. Jude study is exploring the use of an intraventricular reservoir with "LMB-89-like" systemic therapy. Although the intraventricular route provides the added security that the drugs are in fact all getting into the CSF, the treatment results described above suggest that there is no definite therapeutic advantage of the intrathecal route of administration (by lumbar puncture) over the intraventricular route.

The role of cranial radiation for B-cell ALL patients with CNS disease at diagnosis has been somewhat controversial, particularly in light of the excellent results reported with the LMB-89 protocol, which incorporated cranial radiation for patients who were CNS-positive at diagnosis *(10)*. Currently, however, cranial irradiation is not used by the French, German, or United States cooperative groups in the management of patients with CNS disease at diagnosis, primarily because of the excellent results reported with regimens that exclude radiation, such as BFM-90 *(12)*.

3.3. What Is the Optimal Duration of Therapy?

Current successful treatment for B-ALL is generally delivered over 5–8 mo. The question has been raised of whether this duration can be shortened further. One study indicated that

therapy for stage III Burkitt's lymphoma could be shortened to approx 2 m *(18)*, but the final outcome with this approach for B-ALL was inferior to results with longer treatment durations. The group B and group C components of the LMB-89 regimen are generally delivered over 5 and 8 m, respectively *(10)*. In a current FAB collaborative study, one of the randomization questions being addressed is whether the maintenance phases of the LMB-89 regimen can be safely shortened.

3.4. What Is the Role of Various Supportive Care Tools?

3.4.1. Uricolytic Agents

The metabolic abnormalities and subsequent renal failure (sometimes requiring dialysis) that can be associated with the tumor lysis syndrome are significant complications in the management of Burkitt's lymphoma and B-ALL. The use of uricolytic agents, such as uricozyme or its recombinant, rasburicase, are of great value in the management of Burkitt's lymphoma and B-ALL *(39)*. They result in a precipitous drop in the serum uric acid level by converting it to a more soluble form, allantoin. This not only reduces the risk of renal dysfunction and the need for dialysis, but also facilitates the delivery of planned chemotherapy. In this regard, the investigators in the United Kingdom suggested that the inferior result they observed in their LMB-89 trial may have partly resulted from the lack of a uricolytic agent in the management plan *(40)*. These compounds, which have been used for many years in France, are currently under investigation in the United States.

3.4.2. Growth Factors

The use of colony-stimulating factors (CSF) in B-ALL management has generated considerable controversy. In the NCI study of CODOX-M/IVAC, a randomization was performed to test the use of granulocyte/macrophage (GM)-CSF *917)*. Administration of the growth factor was not associated with a reduction in neutropenia, but it was associated with a reduction in the incidence of bacteremia. Thus, the protocol was amended in 1994 to provide GM-CSF to all patients. In a randomized trial testing G-CSF with COPAD-M courses *(41)*, the growth factor was associated with a reduction in the duration of neutropenia but not with an improvement in EFS. A current St. Jude study is attempting to shorten the interval between courses of chemotherapy by using G-CSF in the context of LMB-89-type therapy. If successful, this approach would provide a means of intensifying therapy without increasing drug dose and thus may lead to an improvement in outcome.

3.5. What Is the Recommended Approach to Managing Relapse?

The prognosis for children in relapse following the diagnosis of advanced-stage Burkitt's lymphoma or B-ALL is generally considered to be quite poor, primarily because of the highly intensive therapy they receive initially. The most widely accepted approach to the management of relapse is to determine first whether or not the patient has chemosensitive disease by using any one of a number of intensive multiagent regimens devised for non-Hodgkin's lymphoma. Examples of recently studied combinations include ICE *(26)* (ifosfamide, carboplatin, etoposide), DHAP *(42)* (dexamethasone, high-dose cytarabine, cisplatin), ifosfamide/etoposide, and MIED (high-dose methotrexate, ifosfamide, etoposide, dexamethasone). If the patient is found to have chemosensitive disease, an intensification phase including hematopoietic stem cell rescue is considered *(43–46)*, although the specifics of this option are controversial. There are limited published data on the role of bone marrow transplantation (BMT; autologous or allogeneic) in relapsed Burkitt's lymphoma and B-ALL; however, many investigators would consider an allogeneic strategy in patients with bone marrow involvement, either at diagnosis or at relapse. The European Lymphoma Bone Marrow Transplant Registry has reported the successful salvage of patients with relapsed advanced-stage Burkitt's lymphoma using autologous BMT, but they point out that with more modern intensive initial treatment, autologous BMT strategies may not be as effective *(46)*. They suggest the need for trials examining immunotherapeutic strategies including the potential graft-versus-lymphoma effect that may be associated with an allogeneic BMT but has yet to be clinically demonstrated in patients with B-ALL.

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CHEMOTHERAPEUTIC STRATEGIES

II

ACUTE MYELOID LEUKEMIA IN CHILDREN

H

17 Treatment of Acute Myeloid Leukemia in Children

URSULA CREUTZIG

CONTENTS

1. INTRODUCTION

Acute myelogenous, or myeloid, leukemia (AML) in children represents approx 20% of the acute leukemias. With some minor exceptions, the biology of AML is similar in children and adults. Although AML is much more resistant to chemotherapy than acute lymphoblastic leukemia (ALL), treatment results in childhood AML have improved considerably over the last 20 yr (Fig. 1) *(1,2)*. With intensive induction chemotherapy, 80–90% of children achieve complete remission (CR), which translates into a long-term disease-free survival (DFS) in as many as 50–60% of patients. The strategy of remission induction with the aim of restoring the normal bone marrow function, thus achieving CR, is widely accepted, whereas the options for postremission therapy are still controversial.

Allogeneic stem cell transplantation (allo-SCT) in first CR has yielded similar results in the hands of different study groups, with generally longer DFS than obtained with intensive postremission chemotherapy *(3)*. It therefore may be considered an option for postremission treatment in patients with an HLA-matched sibling. Ablative therapy supported by autologous (auto-) SCT in first CR is another feasible approach, although it carries the obvious risk of reinfusion of leukemic blast cells *(4)*.

A correct diagnosis is required to ensure adequate therapy and is especially important when one is applying risk-adapted treatment strategies. Furthermore, reliable comparisons of different therapy strategies have to be based on common diagnostic or exclusion and inclusion criteria. This chapter outlines the different treatment strategies used in the management of childhood AML and discusses the advantages and disadvantages of each.

2. DIAGNOSIS

2.1. Clinical Manifestations

The first symptoms of AML may be uncharacteristic and include anemia, fever, infection, and bleeding manifestations. Leukemic infiltration of the bone marrow causes a reduction in number and activity of all three hematopoietic cell lines. Extramedullary leukemic infiltrations affect the liver, spleen, and lymph nodes in one-third of the patients. Central nervous system (CNS) involvement [>5 leukemic blasts/mm^3 of cerebrospinal fluid (CSF) or clinical or radiologic signs of intracerebral leukemic infiltration] is found in 5–10% of children with AML. Infiltration of the skin, especially in monocytic

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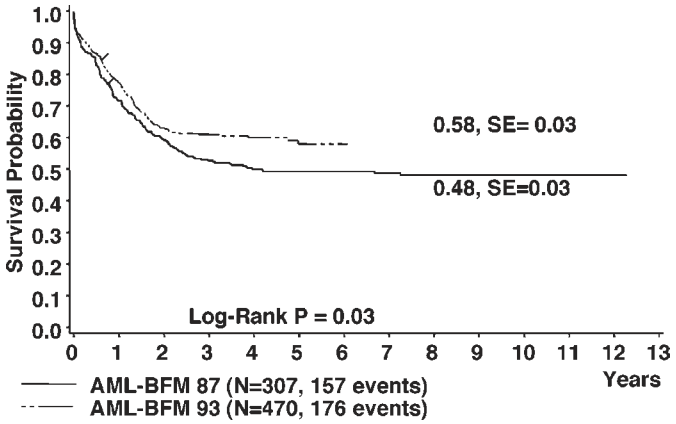


Fig. 1. Estimated probability of survival (± SE) in study AML-BFM 87 compared with study AML-BFM 93.

leukemias, may be important in terms of disease control, because leukemic blasts from these sites may reseed in the bone marrow and eventually produce hematologic relapse.

2.2. Morphologic and Cytochemical Classification

AML is a morphologically heterogeneous disease that may involve several hematopoietic cell lines: granulopoietic cells, monocytes, erythroblasts, and even megakaryocytes. Its classification according to criteria of the French–American–British (FAB) Cooperative Group *(5,6)* is based mainly on cytomorphologic features. Current definitions of the FAB group include 10 different subtypes of AML: granulocytic leukemias (M1, M2, and M3), myelomonocytic and monocytic leukemias (M4 and M5), erythroleukemia (M6) *(5)* supplemented by the variant form of M3 (M3v), the M4 subtype with abnormal eosinophils (M4Eo), acute megakaryoblastic leukemia (M7), and minimally differentiated acute myeloid leukemia (M0) *(6–8)*.

Myelodysplastic syndrome (MDS) occurs mainly in older people and is characterized by an ineffective and dysplastic hematopoiesis in one or more cell lines, as well as by a high risk of leukemic transformation. Differential diagnosis of MDS vs AML is important for therapeutic and prognostic reasons. MDS is rare in children, accounting for only 1–9% of all leukemias diagnosed in this age group *(9,10)*, although many such cases may have been overlooked in the past.

3. THERAPY

3.1. General Aspects of Treatment

The major aim of therapy is to eradicate the leukemic clone, with subsequent restoration of normal hematopoiesis. The first end point to evaluate treatment response is the blast cell count in bone marrow on d 15. According to results of the AML-BFM studies, CR rate and DFS were significantly better for children with a substantial blast cell reduction on d 15 (<5% blasts) compared with those with >5% blasts *(11)* although special practical knowledge is required to assess hypoplastic bone marrows. A complete response by criteria of the National Cancer Institute requires <5% blasts in the bone marrow, with a marrow cellularity of >20% and at least 1500/mm^3 of circulating neutrophils with a duration of response of at least 4 wk *(12)*.

After induction therapy, further intensive treatment is needed to eradicate minimal residual disease (MRD), defined as any remaining leukemic cells not detectable by morphologic criteria. More sensitive immunologic and molecular genetic methods *(13,14)* promise to increase the sensitivity of MRD detection, thus improving definitions of CR, but this potential requires further evaluation in clinical studies. In addition, the significance of the persistence of a leukemic clone in AML with regard to the probability of relapse remains controversial. One exception may be the AML-M3 subtype, whose persistence in cases with a *promyelocytic leukemia (PML)-retinoic acid receptor (RAR)*α rearrangement indicates a high risk of relapse *(15,16)*.

Induction treatment, designed to induce marrow aplasia and render the leukemic clone undetectable, is usually far more intensive in childhood AML than in ALL. Induction therapy is followed by postremission phases that are applied to destroy residual blasts in the bone marrow or at other sites. The duration and the optimal type of postremission therapy remain to be established. Generally, intensive chemotherapy courses termed consolidation and/or intensification courses are administered together with CNS prophylaxis with or without a less intensive maintenance chemotherapy. Allogeneic or autologous bone marrow transplantation (BMT) may be included as another form of intensification (Table 1). The differentiating agent all-*trans*-retinoic acid (ATRA) provides special treatment for patients with AML-M3, inducing cell differentiation and maturation instead of cell destruction *(17)*.

Acute management and supportive care are required during all treatment phases, especially during the first few days and weeks of intensive therapy.

With recent improvements in AML treatment results, the balance between treatment intensity and toxicity has become more important than in the past, warranting trials of risk-adapted therapy (*see* Section 3.7.).

3.2. Remission Induction Therapy

Induction therapies aim to achieve CR and improve long-term results. Generally, this can be accomplished with a single 7-d course of cytarabine (ara-C) and 3 d of anthracyclines. Induction regimens vary in different studies by the manner of administration, either short-term or continuous infusion of ara-C (100–200 mg/m^2/d for 7 d) in combination with daunorubicin (45–60 mg/m^2/d for 3 d). Some regimens include additional drugs as well, such as 6-thioguanine or etoposide (Table 1).

Currently, most of the pediatric AML studies attempt to induce remission with one or two courses of the short and intensive therapy described above. The Cancer and Leukemia Group B (CALGB) study in adults *(18)* demonstrated that ara-C administration for 7 d was superior to a 5-d treatment with the same agent. By comparison, ara-C administration beyond 7 d was too toxic and did not raise the remission rate *(19)*. New data on adults receiving high-dose (3 g/m^2) vs standard-dose ara-C during induction showed a longer remission duration in the high-dose group *(19)*; however, toxicity was extremely high. Daunorubicin produced the best results when given over 3 d at 60 mg/m^2 *(20,21)*. Introduction of the ADE induction regimen [ara-C, daunorubicin (60 mg/m^2 × 3), etoposide] in the AML-BFM 83 trial resulted in a major improvement in long-term results in childhood AML *(22)*.

Table 1
Treatment Strategies and Outcome in Representative Pediatric AML Trials

Study/accrual	*Induction therapy (d)*	*CNS prophylaxis*	*Postremission therapy*	*BMT*	*Patient age (yr)*	*CR rate (%)*	*5-yr pDFS (SE) (%)*	*5-yr pDFS (SE) in subgroups[a] (%)*	*5-yr pEFS (SE) (%)*	*5-yr pSurvival (SE) (%)*
POG-8821										
1988–93 *(51)*	DNR, ara-C, TG (3 + 7 + 7) +HD-ara-C (6)	IT ara-C	VP/AZ, HD-ara-C (6), DNR + ara-C + TG ISC (6 mo)	Allo-BMT, auto-BMT, vs. ISC	649 (<21)	85[b]	40 (at 3 yr)	Allo-BMT 52 (8) Auto-BMT 38 (6) ISC 36 (6) (at 3 yr)	34 (3) (at 3 yr)	42 (3) (at 3 yr)
CCG-2891										
1989–93 *(2)*	DCTER x 2 (2 x 4) intensive vs. standard timing	IT ara-C	DCTER x 2+ HD-ara-C/Asp x 2 + ISC (4 mo)	Allo-BMT, auto-BMT vs. ISC	589 (<21)	74	No data	Intensive timing 55 (8), Standard timing 37 (8) (at 3 yr)	Intensive timing 42 (7), Standard timing 27 (7) (at 3 yr)	Intensive timing 51 (7), Standard timing 39 (7) (at 3 yr)
NOPHO-AML-93										
1993 *(29)* (updated)	ara-C, VP, DOX, TG x 2 (5)	IT MTX	HD-ara-C (± Mitox or VP) x 4	Allo-BMT, auto-BMT	91[c] (<15)	77	No data		61 (6)	
AIEOP										
1987–90 *(52)*	DNR, ara-C (3 + 7), DNR, ara-C (2 + 5)	IT ara-C + P	DNR, ara-C, TG ISC (9 mo)	Allo-BMT, auto-BMT vs. ISC	161 (<14)	79	31 (5)	Allo-BMT 51 (13), Auto-BMT 21 (8), ISC 27 (8)	25 (4)	42 (4)
MRC AML 10										
1988–95 *(30)*	DNR, ara-C, TG x 2 vs. DNR, ara-C, VP x 2 (10 + 8)	IT ara-C, MTX, P	MACE + MidAC	Allo-BMT, auto-BMT vs. Stop	359 (<14)	92	52 (5)	Auto-BMT 68, Stop 48	49 (5)	58 (5)
BFM-93										
1993–98 *(37)*	DNR or IDR, ara-C, VP (3 + 8 + 3)	IT ara-C + CRT	6-wk consolidation, 7 drugs + HD-ara-C (6) Mitox or VP x 2	(Allo-BMT)	470 (<17)	82	62 (3)		51 (2)	60 (3)

Abbrevations: CR, complete remission; *p*, probability; DFS, disease-free survival; EFS, event-free survival; DCTER, dexamethasone, ara-C, TG, VP, DNR; ara-C, cytarabin; HD, high dose; ADR, Adriamycin; Asp, asparaginase; AZ, azacytidine; DNR, daunorubicin; IDR, idarubicin; MTX, methotrexate; P, prednisone; VP, VP-16/213 (etoposide); TG, thioguanine; BMT, bone marrow transplantation; allo, allogeneic; auto, autologous; CRT, cranial irradiation; ISC, intensive sequential chemotherapy; IT, intrathecal; MACE, amsacrine, ara-C, VP; MidAC, mitoxantrone, HD-ara-C;

[a] See Stem Cell Transplantation section (allo-SCT/auto-SCT in first CR) for difficulties comparing BMT results.

[b] Includes patients with M2A bone marrow (5–15% marrow blasts).

[c] Non-Down's syndrome patients only.

Important new drugs used in adult AML trials during induction, consolidation, and intensification therapy are idarubicin and mitoxantrone, and although remission rates were higher with idarubicin than with daunorubicin (45 mg/m^2 or 50 mg/m^2 × 3) *(23)*, long-term results did not improve. Arlin et al. *(24)* demonstrated a higher CR rate after a single induction course with a mitoxantrone (12 mg/m^2 × 3) based regimen compared with the standard regimen, which included daunorubicin (45 mg/m^2 × 3). However, the lack of equivalence of drug doses was a major criticism of this trial *(25)*.

In the pediatric trial AML-BFM 93, idarubicin (12 mg/m^2/d × 3) was compared with daunorubicin (60 mg/m^2/d × 3) during induction. Results showed similar rates of CR and survival; however, the extent of blast cell reduction on d 15 in the bone marrow was significantly better with idarubicin compared with daunorubicin, indicating at least a slightly better antileukemic effect *(26)*. A second induction course with identical or other drugs is often necessary to achieve remission and is given in many trials. Büchner et al. *(20,21)* demonstrated in adults that an intensive double-induction therapy will improve long-term results.

3.3. Consolidation and Intensification

The term *consolidation therapy* refers to the repeated administration of two or more courses of drugs used in the induction phase. Non-cross-resistant sequential drug combinations are administered during *intensification chemotherapy* cycles to circumvent drug resistance. These cycles may be given for several months or up to >1 yr [e.g., the VAPA study*(27)*] or, as in the Berlin–Frankfurt–Munster (BFM) trials, may include an 8- or 6-wk consolidation with seven different drugs *(22,28)* and two blocks of intensification with high-dose ara-C. In the Nordic Society of Pediatric Haematology and Oncology (NOPHO) AML-93 trial, four intensification blocks of high-dose ara-C were included *(29)*, whereas the Medical Research Council (MCR) Acute Myeloid Leukaemia 10 trial applied two highly intensive courses including mitoxantrone and high-dose ara-C *(30)*. Data from these pediatric studies and from studies in adults as well *(31)* revealed a lower relapse rate after the introduction of intensive chemotherapy with high-dose ara-C during postremission treatment *(32)*. This result provides strong evidence for a dose-response effect of ara-C in patients with AML.

The importance of dose scheduling was demonstrated by the Children's Cancer Group (CCG) 213P study, in which two courses of high-dose ara-C/asparaginase were administered at 7-d intervals, resulting in superior survival rates compared with those obtained with the same treatment given at 28-d intervals *(33)*. The number and intensity of chemotherapy blocks required by children with AML remains controversial (*see* Section 7.1.).

3.4. Stem Cell Transplantation

3.4.1. Allogeneic Stem Cell Transplantation (allo-SCT).

The antileukemic effect of allo- SCT is the result of conditioning with ablative chemotherapy and the immunologic graft-versus-leukemia effect. Matched related allo-SCT was evaluated by the CCG in studies 251 and 213.

Although allo-SCT was significantly better than conventional postremission chemotherapy in the 251 study *(34)*, this result could not be confirmed in study 213 *(35)*. We pursued this issue by performing matched-pair analysis among patients enrolled in studies AML-BFM 83 and 87, obtaining equal results in terms of DFS for 16 children who had or had not undergone matched related allo-SCT *(36)*. This outcome was recently confirmed by data from the AML-BFM 93 study *(37)*. In the St. Jude 1980–1983 study *(38)*, there was also no significant difference in the duration of continuous CR with or without allo-SCT in a program of intensive sequential chemotherapy, but postremission failures resulted more often from bone marrow relapse in the sequential chemotherapy group (23 of 42 patients, 2 deaths in CR) compared with the SCT group (5 of 19 patients with bone marrow relapse, 5 deaths in CR). In the MRC AML10 trial, the 7-yr overall survival rate was 56%, representing substantial improvement on previous trials. Allo- and auto-bone marrow transplantation (BMT) reduced the relapse risk but did not produce a significant survival benefit. Eleven deaths in CCR were associated with BMT *(39)*.

The earlier results notwithstanding, outcome for children receiving allo-SCT in first CR of AML has improved during the last decade. Michel et al. *(40)* attribute this to a decreased risk of transplant-related mortality from 36% between 1979 and 1986 to 3% between 1987 and 1990. The Seattle experience in adult patients, reported by Appelbaum et al. *(41)*, showed a lower relapse rate in the transplanted group compared with the chemotherapy group with use of intensive postremission consolidation and maintenance therapy.

Concerning the preparative regimen, busulfan plus cyclophosphamide is commonly applied in children. Data from the European Group for Blood and Marrow Transplantation (EBMT) comparing this regimen in matched controls treated with cyclophosphamide plus total-body irradiation *(42)* showed identical transplant-related mortality, relapse rates, and DFS. Recently, good results were reported in 31 patients receiving high-dose chemotherapy with busulfan, cyclophosphamide, and etoposide as conditioning agents. The DFS rate was 80% (median follow-up, 30 mo). There were no relapses; all deaths were caused by transplant-related toxicities *(3)*.

The toxicity of SCT includes major organ toxicity, e.g., hepatic venoocclusive disease or interstitial pneumonia, acute graft-versus-host disease (GvHD) clinically manifested as rash, hepatic dysfunction, diarrhea, mucositis or fever. Late toxicity may include growth impairment, endocrinologic disturbances, late cardiotoxicity, and chronic GvHD *(43)*.

Whether or not all patients with AML should undergo matched related allo-SCT in first CR is controversial (*see* Section 7.2.).

3.4.2. Unrelated Donor Transplantation

This procedure is as effective in eradicating leukemic cells as SCT with a related donor. Results in 18 children and young adults receiving T-cell-depleted unrelated donor bone marrow in first or second CR were promising, with 14 patients surviving at 2 yr *(44)*. However, because of the small size of this study and the high risk of acute or chronic GvHD *(45)*, unrelated donor transplantation should not be considered in first CR in children with AML *(46,47)*.

3.4.3. Autologous Stem Cell Transplantation

High doses of chemotherapy and radiotherapy can be administered if rescue by auto-SCT is available. Although less toxic than allo-SCT because of its elimination of the GvHD hazard, auto-SCT has proved less efficacious and carries the risk of reinfusion of leukemic stem cells *(48)*. In vitro purging is an attempt to rid the marrow of these cells. Purging agents in AML have been cyclophosphamide derivates, such as 4-hydroperoxycyclophosphamide (4-HC) *(49)* or mafosfamide *(50)*. Most reports demonstrate a 1–3-yr survival rate of 30–50% for patients in first CR and approx 20% for patients in second CR. Randomized study 8821 of the Pediatric Oncology Group (POG) *(51)* compared the efficacy of auto-SCT and intensive consolidation chemotherapy (ICC). Results for event-free survival (EFS) were equal when analyzed by intent-to-treat (36% ICC vs 38% auto-SCT). The relapse rate was lower (31% vs 58%), but treatment mortality was higher in the auto-SCT compared with the ICC group (15% vs 3%). Similar results were obtained by another study in Europe (AIEOP/LAM 87) for auto-SCT vs postremission chemotherapy, with superior results for children treated with allo-SCT *(52)* (Table 1). It was recently demonstrated by the MRC-10 trial that auto-BMT after four courses of intensive chemotherapy could reduce the relapse rate; however, both morbidity and mortality were increased *(4)*. Moreover, this option was not compared with an additional block of chemotherapy. On the strength of this observation, a delay in autografting was recommended, at least for good-risk groups. Up to now, auto-SCT alone does not appear to have improved cure rates in childhood AML, but better results might be achieved by improving purging and preparative regimens *(53)*.

3.5. Maintenance Therapy

So far, there are no common strategies regarding maintenance therapy. The duration and mode of this phase of treatment are still topics for debate (*see* Section 7.3.).

3.6. CNS Prophylaxis and Therapy

Prophylactic cranial irradiation has not generally been included in therapy protocols for AML in adults and children. Most investigators agree that cranial irradiation will prevent CNS relapses, but its effect on overall remission duration has not been determined *(54)*. Results of ALL trials in children *(55)* and of the AML study 72–75 reported by Dahl et al. *(56)* indicated that cranial irradiation had an impact on the number of CNS relapses but not on overall survival. Most AML studies in children and adults use intrathecal methotrexate or ara-C or a combination of these agents with hydrocortisone. In the AML-BFM studies, cranial irradiation was generally included. AML-BFM 87 tested prospectively whether cranial irradiation could be replaced by late intensification therapy with high-dose ara-C and etoposide *(28)*. Although the results favored cranial irradiation, randomization was stopped early, so that the data were mainly based on the nonrandomized group treated without cranial irradiation after randomization was stopped. Therefore, these findings still have to be confirmed by other pediatric or adult AML trials.

An outstanding controversy is whether inthrathecal and high-dose ara-C or methotrexate are sufficient to control MRD in the CNS or whether cranial irradiation must be added (*see* Section 7.4.).

3.7. Prognostic Factors for Risk-Adapted Treatment Strategies

The main goal of risk factor analysis is to define the individual risk of failure at the time of diagnosis or as soon as possible after the first treatment course. It should be stressed that most prognostic parameters are not independent, and their significance can change when treatment becomes more intensive. In childhood AML, young age is considered an adverse prognostic factor. A poor outcome, especially for infants compared with older children, was reported by Grier et al. *(57)* and Buckley et al. *(58)* and was confirmed in studies AML-BFM-83 and -87 *(11)*. However, this result could be shown only by univariate analysis; statistical significance was lost when the data were examined by multivariate methods. Other factors predicting an unfavorable prognosis were: high white blood cell (WBC) count, unfavorable cytogenetic findings such as monosomy 7 and complex aberrations *(59)*, and FAB types M4 and M5 *(57,58)*. According to the results of the AML-BFM, a high WBC count ($\geq$100,000/mm^3) was associated with an increased risk of early death owing to hemorrhage and/or leukostasis, particularly in patients with AML-M5 and concurrent hyperleukocytosis *(60)*. This feature was also associated with a high risk of nonresponse, but after remission was achieved, DFS was not significantly different in comparison with patients with a lower WBC (<100,000/mm^3, $p = 0.095$ by log-rank analysis) *(11)*. Multivariate analysis revealed a high correlation with other parameters, including a delay in blast cell reduction until d 15.

Because of the biologic heterogeneity of AML, the prognostic significance of a high WBC count varies among the different FAB subtypes, for example, patients with AML-M3 usually present with a low WBC count but have a higher risk of early death by disseminated intravascular coagulation, whereas children with AML-M7 are at a higher risk for treatment failure, even without an increased WBC count. Comparison of studies AML-BFM 78 and 83 showed an improved prognosis for FAB types with predominantly granulocytic differentiation (FAB M1–M4) after intensification of chemotherapy in study 83. Based on the results of studies AML-BFM 83 and 87, two different risk groups (standard and high) could be defined by the use of predominantly pretherapeutic parameters, including FAB type and morphologic findings, such as Auer rods and eosinophils, as well as the blast cell count in bone marrow on day 15 *(11)* (Fig. 2). These risk groups correlate with cytogenetic findings used in other studies to define risk criteria, e.g., in the MRC trial *(61)*). Our data revealed an association between FAB types M1 and M2 with Auer rods, M3, and M4Eo and the favorable cytogenetic features t(8;21), t(15;17), and inv16 (Table 2) *(11,59,62,63)*. Nonetheless, there is still considerable debate about whether risk group assignments are needed to stratify patients for therapeutic options (*see* Section 7.5).

3.8. Therapy Options for Special Patient Groups

3.8.1. Down's Syndrome

Children with Down's syndrome have an approximately 20-fold higher incidence of leukemia than do children without

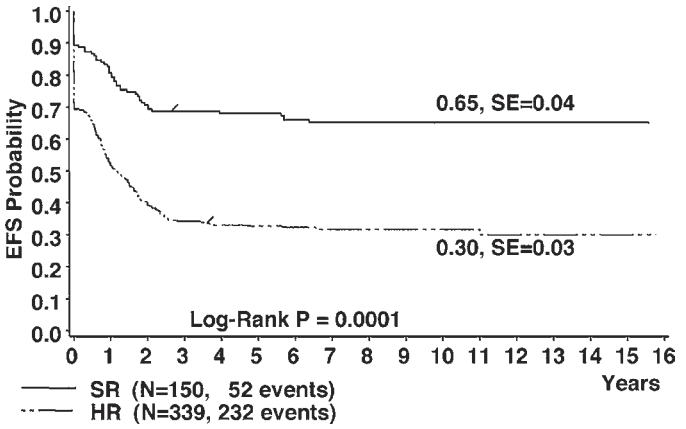


Fig. 2. Estimated probability of event-free survival (EFS ± SE) for patients with standard-risk (SR) versus high-risk (HR) AML in studies AML-BFM 83 and 87.

Table 2
Correlation Between Risk Groups and Karyotypes[a]

	Favorable karyotypes		*Unfavorable karyotypes*		
Risk group[b]	*N (%)*	*pEFS (SE) (%)*	*N (%)*	*pEFS (SE) (%)*	*p-valuec*
Standard risk	43 (62)	58 (8)	26 (38)	73 (9)	0.19
High risk	11 (8)	73 (13)	**125 (92)**	**27 (4)**	**0.001**
CR patients only		*pEDS*		*pDFS*	
Standard risk	41 (65)	61 (8)	22 (35)	86 (7)	0.01
High risk	11 (11)	73 (13)	**86 (89)**	**40 (5)**	**0.02**

Abbrevations: *p*EFS, probability of event-free survival; *p*DFS, probability of disease-free survival; CR, complete remission.

[a] For further details, *see* ref. 61. Data in bold type are statistically significant.

[b] Standard risk: FAB M1/M2 with Auer rods, FAB M3, FAB M4eo, ≤5% marrow blasts on d 15 (except for M3); high risk: all others.

[c] Kaplan-Meier test.

this condition. They also have other unique hematologic features, including a transient myeloproliferative disorder in newborns, which normally disappears spontaneously *(64)*, a peak incidence of AML under 4 yr of age *(65,66)*, the frequent occurrence of a MDS phase preceding the development of AML *(67–69)*, and a high incidence of M7 leukemia evolving from a myelodysplastic stage with or without myelofibrosis, making it difficult to apply standard FAB criteria *(6,7)*. Leukemia diagnosed in young children with Down's syndrome is a unique biologic AML subtype. FAB M7 with more or less than 30% blasts represents the same disease and should be classified as M7-Down *(70)*. This entity can be separated from M7 leukemia in non-Down's syndrome infants with t(1;22) and M7 leukemia in adults with 3q abnormalities. A common progenitor for thrombo- and erythropoietic cells may be affected, as interphase cytogenetic analysis showed clone-specific markers in cells of both lineages *(71)*. It appears that the development of M7-Down is associated with a critical period of pre- or perinatal hematopoiesis in children with Down's syndrome. This interpretation is supported by the occurrence of transient myeloproliferative disorders (TMDs) in neonates with Down's syndrome *(72)* and the observation that most of these leukemias occur within the first years of life.

In contrast to the poor responses and low survival rates among non-Down's syndrome children with FAB M7 leukemia, a high cure rate has been reported in AML patients with Down's syndrome *(68,73)*. The good response to chemotherapy may be attributed to an enhanced intracellular metabolism of ara-C to cytarabine triphosphate (ara-CTP) in Down's syndrome cells *(74)*. Since thrombocytopenia is the predominant symptom during the early stage of M7-Down, petechial bleeding or a decrease in the platelet count demands a bone marrow biopsy, and if the diagnosis of M7 leukemia is confirmed, treatment should be started as soon as possible. However, considering the low relapse rate reported *(68,70,73)* and the high remission rate, overtreatment should be avoided. We recommend standard AML therapy with one cycle of high-dose ara-C without cranial irradiation, owing to the increased sensitivity of the leukemic blasts to ara-C. Because of the young age of Down's syndrome patients, the cumulative dose of anthracyclines should not exceed 240 mg/m^2 *(75)*, and a reduction in doses of daunorubicin, doxorubicin, and idarubicin is recommended. Allogeneic stem cell transplantation in first remission is not recommended. The risk of letal infectious complications in remission can be reduced by careful adherence to supportive care guidelines and follow-up procedures.

3.8.2. Transient Neonatal Myeloproliferative Disorders

Hematologic abnormalities indistinguishable from acute leukemia by clinical and morphologic criteria have been described in neonates with Down's syndrome. In these children, spontaneous remission occurs without specific chemotherapy *(76)*. However, intensive supportive care to reduce hyperviscosity and treatment of sometimes severe coagulation disturbances may be necessary, as may erythrocyte and platelet transfusions. It appears that the existing additional chromosome 21 is the key for the leukemic reaction or the development of leukemia. The leukemic blasts show predominantly morphologic and immunologic features similar to blasts of acute megakaryocytic leukemia (FAB M7 subtype). In some cases, karyotyping shows additional clonal aberrations like trisomy 8, which disappear on clinical remission. In singular cases, in vitro cell cultures reveal normal growth and differentiation in the TMD blasts, in contrast to leukemic blasts of older children with Down's syndrome and non-Down's syndrome patients. These results suggest that the initially proliferating cell clone loses its advantage of growth on maturation, resulting in a spontaneous remission in newborns with Down's syndrome and TMD. However, these children are at higher risk for a malignant transformation during the next 2–4 yr of life, as approximately 20% of them develop AML, hence, close surveillance of this patient subgroup is mandatory.

4. CHEMOSENSITIVITY BY SPECIFIC SUBTYPE

4.1 Acute Promyelocytic Leukemia

Results of cooperative studies have demonstrated high CR induction rates in patients with acute promyelocytic leukemia (APL) treated with ATRA alone or with ATRA and chemo-

therapy *(78)*. The best results have been achieved with simultaneous application of ATRA and chemotherapy *(78)*. Further trials in adults in which idarubicin as the single chemotherapy agent was combined with ATRA supported the thesis that ara-C might be omitted from induction therapy in this disease *(79,80)*. Recent data from the M.D. Anderson and PETHEMA groups suggest that ara-C may also be omitted in consolidation therapy with anthracyclines *(79)*. However, since children do not tolerate higher cumulative doses of anthracyclines, therapy protocols for adults with APL should not be adopted for children without first considering the anthracycline doses.

4.2. AML with Other Specific Karyotypes

Besides the correlation of the rearrangement *PML-RARα* with AML-M3, there are other specific molecular rearrangements associated with a good prognosis. One of them is the translocation t(8;21)(q22;q22), which is found mainly in AML-M2 arising *de novo*. Patients with t(8;21) show the *AML1-ETO* fusion transcript, which in adult studies is associated with a relatively good prognosis and a particularly good response, especially to ara-C *(31,81)*.

Another prognostically favorable balanced translocation is inv(16) with the fusion gene transcript *CBFβ-MYH11*. It has been shown in adults that treatment with high-dose ara-C induced prolonged remission in these patients *(31,82)*. Recently, Seymour et al. *(83)* demonstrated that, similar to *de novo* AML with inv(16), secondary diseases may also benefit from treatment with an escalated dose of ara-C. These observations in patients with APL and in patients with specific favorable karyotypes may lead to drug-adjusted therapies based on cytogenetic results.

Different drugs may be mandatory for acute monoblastic leukemia or AML with 11q23 aberrations *(11,59,62)*. Acute monocytic leukemia was generally regarded to be prognostically unfavorable in both children and adults *(84,85)*, yet monoblasts may be more sensitive to specific drugs than other AML subtypes, as reported in the past for VP16 (etoposide) *(86)*. In a phase I trial in relapsed patients, CR rates with VP-16 ranged up to 20%, compared with 35–70% for the FAB subtypes M5 and M4 *(87)*.

Recently, in vitro studies with the methyl-thiazol-tetrazolium (MTT) essay comparing the resistance profiles of untreated leukemic cells from 18 children with AML-M5 with those of 84 children with non-M5 AML were performed. FAB M5 samples were more sensitive to etoposide and 2-chlorodeoxoyadenosine (2-CDA), cytarabine, and (to a lesser extent) daunorubicin and doxorubicin. The cells were also sensitive to drugs commonly used to treat ALL, vincristine and L-asparaginase *(88)*, and were particularly resistant to glucocorticoids *(89)*.

New clinical studies with 2-CDA have shown activity in AML patients *(90)*, especially in children with M5 leukemia (R.C. Ribeiro, personal communication). In vitro studies have shown that AML-M5 was three times more sensitive to methotrexate than were non-M5 samples ($p = 0.06$) and twice as resistant as pre-B-ALL samples after short-term exposure. During long-term continuous exposure, AML M5 cells were even more sensitive to methotrexate than either non-M5 samples or pre-B-ALL samples *(91)*.

These results suggest that patients with acute monoblastic leukemia (FAB M5) may benefit from treatment with alternative drugs, particularly methotrexate. A window study of long-term (36-h) exposure to methotrexate is under way in the current BFM trial in AML patients in first relapse.

5. TOXICITY AND SUPPORTIVE CARE

5.1. Early Death Owing to Leukostasis and Hemorrhage

The portion of children with AML failing to achieve CR ranges between 10 and 25%. Approximately one-half of these patients have refractory leukemia; the others die of early fatal complications owing to leukostasis, hemorrhages, or infections, before a treatment response can be achieved. After CR induction, the rate of fatal complications is much lower (e.g., 4% in the AML-BFM studies). A high risk of early death due to hemorrhage or leukostasis *(39,60)* is closely associated with mono- or myelomonocytic leukemia (FAB M4 or M5 subtype) and hyperleukocytosis (WBC ≥ 100,000/mm^3). Therefore, strategies to reduce these early leukemia-related complications have been established.

Leukostasis, defined as the vascular accumulation of leukemic cells *(92)*, is associated with a high circulating blast count and with a high viscosity, especially in cases with high volumes of myeloblasts and monoblasts. The clinical manifestations of leukostasis include neurologic symptoms (confusion and drowsiness or cardiopulmonary signs, such as dyspnea). Emergency care with intensive monitoring, careful hydration with concurrent urine alkalization, and administration of allopurinol is recommended. Exchange transfusion can be life-saving. Blood exchange is more advantageous than leukopheresis, particularly in young children, because in addition to blood cell reduction, metabolic imbalances and hemostatic disturbances can be corrected and greater shifts in volume prevented. To prevent further proliferation of blast cells, immediate administration of hydroxyurea or ara-C is recommended.

Early fatal *hemorrhages* can develop in the context of leukostasis or independently by spontaneous or chemotherapeutically induced blast cell lysis. The most significant coagulation parameter for predicting fatal hemorrhage was a low plasminogen level *(93)*. Therapeutic guidelines for coagulation disturbances in AML include exchange transfusion in patients with extremely high WBC counts and fresh frozen plasma in those with isolated plasmatic hemostatic disturbances. Platelet transfusion is indicated, in cases of thrombocytopenic and/or thrombocytopathic hemorrhage. In APL, the risk of hemorrhage induced by cell lysis could be reduced by ATRA, which can induce blast cell differentiation in these patients *(94)*.

5.2. Other Acute Complications

Renal insufficiency may occur in patients with extreme cytolysis. In these cases forced diuresis, urine alkalization, and cautious cytoreduction is indicated. Hemodialysis is mandatory in patients with renal insufficiency or uncontrollable metabolic disturbances.

5.3. Toxicity of Chemotherapy

Treatment of children with AML is often associated with acute and chronic complications. Intensive induction therapy

results in severe bone marrow aplasia associated with infections and hemorrhages from thrombocytopenia. To prevent fatal infection, one should consider antimicrobial prophylaxis consisting of nonabsorbabal antibiotics and co-trimoxazole, together with disinfection of skin and mucous membranes. Fever of unknown origin in the granulocytopenic phase of AML should be attributed to bacterial or fungal sepsis. The toxic side effects of individual chemotherapeutic agents include, among others, cardiomyopathy caused by anthracyclines, neurotoxicity caused by vincristine and high-dose ara-C, and hepatotoxicity caused by mitoxantrone and ara-C. Because of the increasing cure rates in children with AML, late effects of chemotherapy have to be considered in the design of new therapy protocols.

6. FUTURE PROSPECTS AND NEW TREATMENT MODALITIES

Survival rates in children with AML have improved over the last two decades, from <10% to >50%. This advance was made possible by improved intensified chemotherapy and supportive care. Comparison of the treatment strategies and results (Table 1) indicate that successful treatment regimens should include the following elements: intensive induction courses with anthracyclines of an adequate dosage, consolidation and/or intensification with one or more courses of high-dose ara-C, some kind of CNS prophylaxis (which may include cranial irradiation), and at least several months of intensive or maintenance chemotherapy. Stem cell transplantation with an appropriately matched HLA-compatible sibling donor is another possibility for patients in first remission. Treatment of AML involves greater risks and is more difficult than treating ALL, owing to a higher risk of initial life-threatening complications, such as hemorrhages and/or leukostasis, and to the fact that most of the drugs capable of eradicating myelogenous leukemic cells will also destroy normal residual myelopoietic cells.

Further intensification of chemotherapy for AML can be expected in only a limited way; thus it is crucial that new strategies will be developed for the management of AML.

6.1. Optimizing Treatment by Considering Pharmacokinetic Parameters and the Interaction of Cytostatic Drugs

An in vitro cellular pharmacokinetic model for the nucleotide ara-CTP, in which cellular uptake and intracellular phosphorylation were determinants for the cytostatic effect of ara-C, showed marked differences in ara-CTP retention among the morphologically classified types of leukemia. The cellular accumulation of ara-CTP is similar in all cell types. The decrease of ara-CTP, however, is significantly more rapid in T-ALL and AML compared with non-T-ALL, providing a pharmacokinetic rationale for continuous infusion of ara-C in these subgroups as an alternative to the intensification by high-dose ara-C schedules *(95)*. Methotrexate resistance in AML is associated with impaired polyglutamylation resulting from low folypolyglutamate synthetase and high folypolyglutamate hydrolase activity. As the resistance could be overcome by continuous exposure to high-dose methotrexate or novel antifolates, continuous exposure may be another approach to circumvent methotrexate resistance in AML cells *(91)*.

6.2. New Drugs

6.2.1. 2-Chlorodeoxyadenosine (2-CDA)

This agent has shown significant antileukemic activity in patients with recurrent or refractory AML. In 32 of 36 adult patients, 2-CDA given at 5–21 mg/m^2 after intensive prior therapy eliminated circulating blasts, although higher doses were associated with prolonged myelosuppression and degenerative neuropathy *(96)*. In a phase II study of 2-CDA (8.9 mg/m^2/d for 120 h), given as a single agent in children with newly diagnosed AML, 6 of 22 patients achieved CR and 7 a partial remission, for an overall response rate of 59% *(90)*.

6.2.2. Fludarabine

Commonly used as a means of enhancing ara-C activity, fludarabine is often administered at doses of 25 mg/m^2/d (5 d) in combination with high-dose ara-C (5 d) and granulocyte colony-stimulating factor (G-CSF) (from d 1 until neutrophil recovery) as the so-called FLAG regimen (fludarabine, ara-C, G-CSF). FLAG has been evaluated in small studies of patients with relapsed or refractory AML. Reported CR rates have varied from 50% in 22 patients (relapsed/refractory AML) *(97)* to 68% in 19 patients with relapsed AML, to all 4 patients with refractory AML *(98)*. FLAG or FLANG (FLAG + mitoxantrone, 10 mg/m^2, after ara-C) induced CRs of 62% in 29 patients with poor-risk or refractory *de novo* AML and 54% in 22 patients with secondary AML *(99)*.

6.3. New Treatment Methods Based on Hematopoietic Growth Factors and Differentiating Agents

Since hematopoietic growth factors [granulocyte-macrophage (GM)-CSF and G-CSF] stimulate the proliferation of clonogenic leukemic cells in vitro *(100,101)*, the use of growth factors in leukemic patients has been regarded with caution. However, several studies showed that CSFs could decrease the duration of neutropenia modestly if such therapy began soon after the end of AML induction therapy; the benefits of this drug regarding the duration of hospitalization, incidence of severe infection, and long-term outcome were less clear *(102)*. Pui et al. *(103)* stated that G-CSF treatment had some clinical benefit in children with ALL, showing fewer documented infections and a shorter hospitalization after induction treatment. Administration of G-CSF or GM-CSF to adult AML patients after intensive induction and postremission chemotherapy accelerated the granulocytic recovery significantly without adverse effects on CR duration and survival *(104–106)*. The use of thrombopoietic growth factors is not yet clear in AML. In two studies, patients required fewer platelet transfusions if the second course was augmented with interleukin-11 (IL-11). However, when used in AML patients after a 7-d standard induction, thrombopoietic growth factor did not accelerate platelet recovery. In view of its potential adverse effects in AML patients, this drug should be studied carefully *(107)*.

In addition to its supportive effect, GM-CSF was tested for its "priming effect" on antileukemic chemotherapy. The aim is to recruit cells into sensitive phases of the cell cycle to increase their sensitivity to cycle-active chemotherapeutic agents. This

could be demonstrated in vitro *(101)*, but the first results in AML patients treated with chemotherapy plus GM-CSF have been controversial *(108,109)*. Recently, Thomas et al. *(110)* reported on the multicenter randomized placebo-controlled etoposide, mitoxantrone, and cytarabine (EMA)91 trial in adults with GM-CSF administered between the two sequences of EMA chemotherapy and during the second sequence. The goal was to increase therapeutic efficacy by potentially increasing leukemic cell recruitment into the S-phase of the cell cycle before the second sequence. This rationale did not translate into significantly higher disease-free and overall survival rates. Cell cycle studies showed an increased recruitment of cells into the S-phase between d 4 and d 8 in the GM-CSF group compared with the placebo group ($p = 0.006$), but this observation was not significantly related to prognosis in this cohort of patients. GM-CSF might marginally increase the efficacy of sequential chemotherapy without increasing its toxicity in the absence of any detectable relationship between this effect and observed leukemic cell recruitment into the cell cycle.

Wide use of the differentiation inducer ATRA has improved the prognosis of APL considerably during the last 10 years (*see* Chap. 18). The molecular basis for this kind of therapy is explained by the reduced retinoid acid sensitivity of nuclear receptor corepressor binding to *PML-RARα*. This fusion protein inhibits the dissociation of the histone deacetylase corepressor complex. Since the ATRA sensitivity of the corepressor association with the *PML-RARα* is lower than with wild-type *RARα*, pharmacologic but not physiologic concentrations of ATRA promote the dissociation of the corepressor, association of the coactivator (SRC-1) with histone acetylation activity, and thereby further transcription, leading to differentiation *(111,112)*. Clinical studies to develop differentiating agents as a new approach to the treatment of specific subgroups of non-APL patients are under way *(113)*. New agents to be tested include butyrates, which can induce the differentation of non-APL cell lines.

With further knowledge of these factors, it may become possible to control the growth and differentiation of all leukemic blasts, in the manner already adopted for stimulation of APL with the differentiating agent ATRA.

6.4. Immunotherapy

Results of nonspecific immunotherapy for AML, using agents such as *Bacillus Calmette-Guérin*, methanol extraction residual of tubercle bacillus, *Corynebacterium parvum* or levamisole, have not been convincing *(114)*. In the 1980s, in vitro and preclinical studies showed that IL-2 had significant antitumor effects. Clinical trials of continuous infusions of IL-2 following auto-BMT in patients with hematologic malignancies showed a significant immunomodulatory effect, which so far has not translated into improved survival. Moreover, it should be considered that significant IL-2-induced systemic toxicity can be expected in about 30% of patients *(115)*. In addition, IL-2 has been used as maintenance therapy in AML to promote immune-mediated eradication of residual leukemic cells *(116)*, and with limited success in patients with relapsed AML who have low blast counts or have entered second CR *(117)*.

6.5. Antibody-Targeted Chemotherapy

Antibody-targeted chemotherapy with gemtuzumab ozogamicin (Mylotarg™, Wyeth Laboratories, PA) is now under investigation. The CD33 antigen is expressed on normal and leukemic myeloid colony-forming cells and on most AML cells, but not on normal pluripotent hematopoietic stem cells. Gemtuzumab ozogamicin is an antigen-targeted chemotherapy agent consisting of an antigen-CD33 antibody linked to calicheamicin, a potentially cytotoxic agent. A phase I study of this drug performed in adults with relapsed or refractory AML resulted in blast cell reductions of <5% in 8 (20%) of the 40 patients. Blood counts returned to normal in three patients. Fever and chills were the most common toxic effects. These promising results show that an immunoconjugate targeted to CD33 can selectively reduce the number of AML blasts in some patients *(118)*.

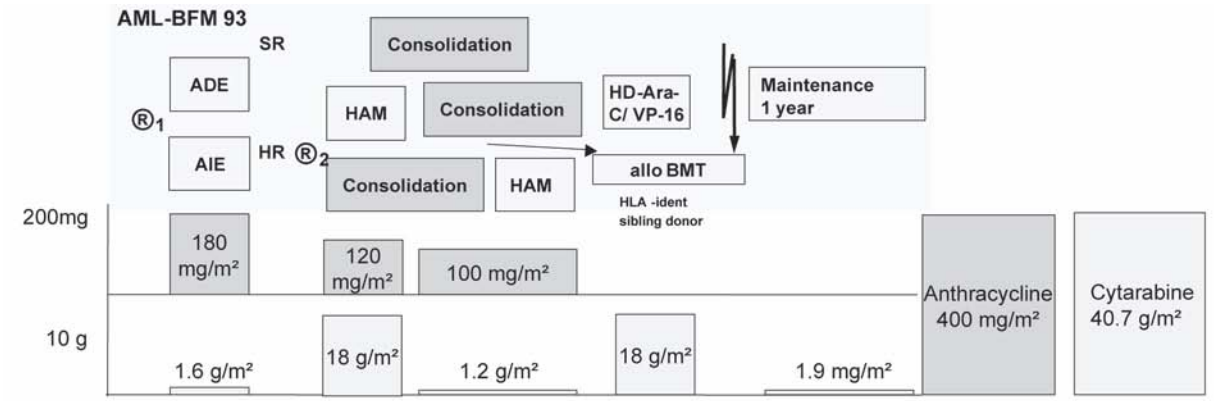
6.6. Monitoring Residual Disease

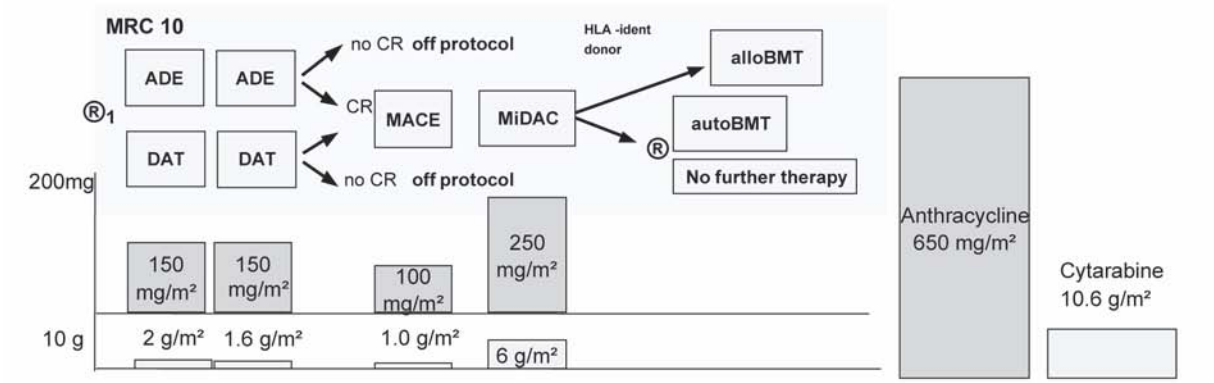
Since cytogenetic/molecular markers and immunologic methods can be used to identify residual disease at clinical remission, a definition of complete remission on the molecular or immunologic level is possible. Therefore, monitoring of residual disease may be helpful in reaching decisions on the cessation of therapy. Two methods are used to detect MRD. Polymerase chain reduction (PCR) analysis to detect abnormal gene rearrangements or fusion genes yields a high sensitivity (up to 10^{-5}), but only about 30% of AML-specific markers can be detected by this approach, and results are only qualitative. Real-time PCR offers both the advantage of higher sensitivity and the possibility of quantification.

By immunophenotyping with flow cytometry, about 80% of patients show informative immunophenotypes, but the sensitivity of this method was much lower (10^{-3} to 10^{-4}) than that of real-time PCR. On the other hand, difficulties may arise in distinguishing between malignant blasts and rapidly proliferating progenitor cells in the regenerating bone marrow. Furthermore, an antigen loss during treatment may occur, complicating the identification of the leukemic blasts. In addition, the clinical significance of MRD needs to be established, because so far, its relevance to patient management is not known. As shown in patients with APL and a detectable *RARα* rearrangement *(119)*, the persistence of an abnormal gene rearrangement might be indicative of later relapse. On the other hand, the *AML1-ETO* rearrangement found in cases with the translocation 8;21 *(120)* was seen in patients with long-term remissions *(121)*.

Recently developed RT-PCR methods can amplify the *PML-RARα* fusion gene product with increased sensitivity, to the level of two leukemic cells in 10^6 normal cells *(122)*. Five of 11 patients in long-term remission who were negative by standard RT-PCR tested positive with the "hot-start" PCR. This indicates that a low level of *PML-RARα* expression may be possible in "clinically cured" patients, so that the sensitivity of the MRD detection methods has to be taken into account.

Apart from these problems of appropriate standardization and correct interpretation, first clinical experiences with salvage therapy given at the time of first molecular relapse have been reported in 14 patients with *PML-RARα*-positive APL. Second molecular remission was obtained with ATRA and


AML-BFM 93
SR
ADE
AIE
HR
HAM
Consolidation
Consolidation
HD-Ara-C/ VP-16
Maintenance 1 year
allo BMT
HLA -ident sibling donor
Consolidation
HAM
200mg
10 g
180 mg/m²
120 mg/m²
100 mg/m²
1.6 g/m²
18 g/m²
1.2 g/m²
18 g/m²
1.9 mg/m²
Anthracycline 400 mg/m²
Cytarabine 40.7 g/m²


MRC 10
ADE
ADE
DAT
DAT
no CR off protocol
CR
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HLA -ident donor
alloBMT
autoBMT
No further therapy
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200mg
10 g
150 mg/m²
150 mg/m²
100 mg/m²
250 mg/m²
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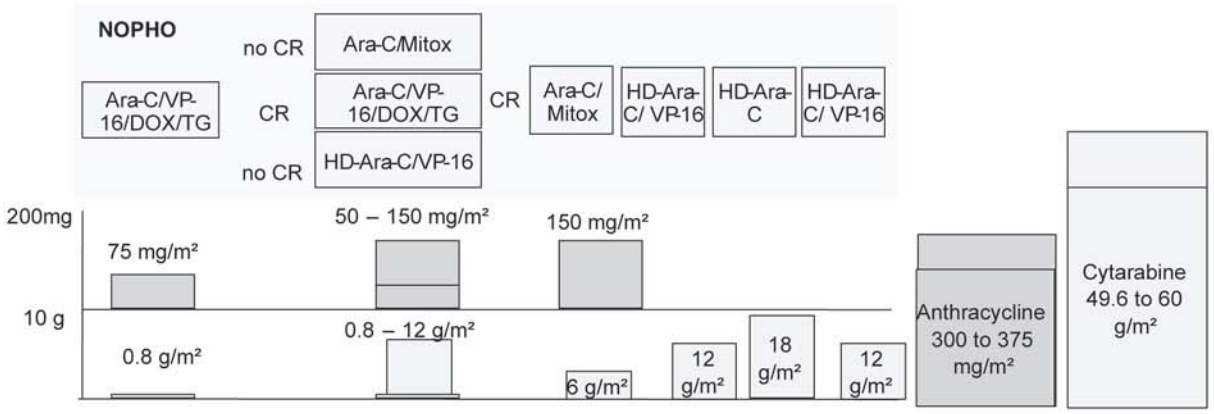
6 g/m²
Anthracycline 650 mg/m²
Cytarabine 10.6 g/m²


NOPHO
Ara-C/VP-16/DOX/TG
no CR
Ara-C/Mitox
CR
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no CR
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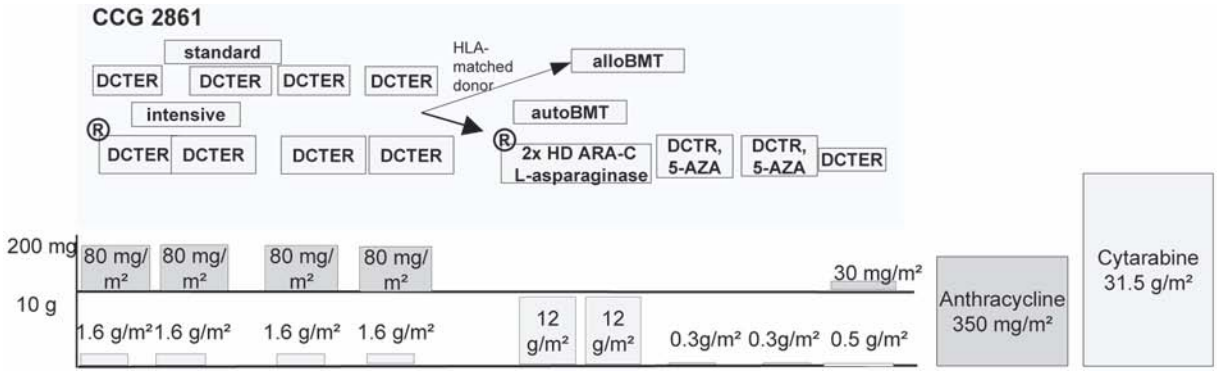
CR
Ara-C/ Mitox
HD-Ara-C/ VP-16
HD-Ara-C
HD-Ara-C/ VP-16
200mg
10 g
75 mg/m²
50 – 150 mg/m²
150 mg/m²
0.8 g/m²
0.8 – 12 g/m²
6 g/m²
12 g/m²
18 g/m²
12 g/m²
Anthracycline 300 to 375 mg/m²
Cytarabine 49.6 to 60 g/m²


CCG 2861
standard
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DCTER
2x HD ARA-C L-asparaginase
DCTR, 5-AZA
DCTR, 5-AZA
DCTER
200 mg
10 g
80 mg/m²
80 mg/m²
80 mg/m²
80 mg/m²
30 mg/m²
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1.6 g/m²
1.6 g/m²
1.6 g/m²
12 g/m²
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Anthracycline 350 mg/m²
Cytarabine 31.5 g/m²

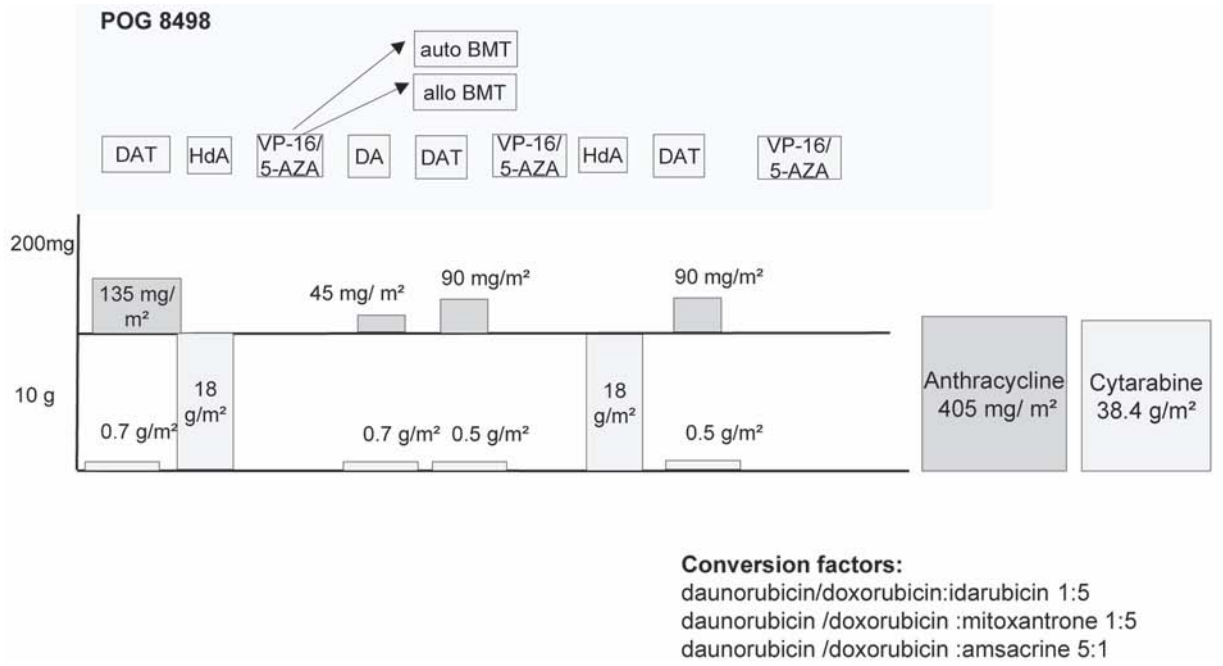

Fig. 3. Overview of regimens and cumulative doses of ara-C and anthracyclines in five pediatric studies. CR, complete remission; ara-C, cytarabin; HD, high dose; DOX, doxorubicin; 5-AZA, 5-azacytidine; DNR, daunorubicin; HAM, HD-ara-C/mitoxantrone; MTX, methotrexate; P, prednisone; R, randomization; SR, standard risk; HR, high risk; VP, VP-16/213 (etoposide); TG, thioguanine; BMT, bone marrow transplantation; allo, allogeneic; auto, autologous; i.th., intrathecal; ↯, cranial irradiation; ADE, ara-C/daunorubicin/etoposide; AIE, ara-C/idarubicin/etoposide; DA, DNR/ara-C; DAT, DNR/ara-C/thioguanine; DCTER, dexamethasone/ara-C/TG/ VP/DNR; MACE, amsacrine/ara-C/VP; MiDAC, mitoxantrone/HD-ara-C.

chemotherapy in 12 patients, 10 of whom remained in CR. These results were significantly better than those for a previous group of patients with hematologic relapse who received the same treatment. The study suggests that early administration of salvage therapy is advantageous in APL *(123)*.

As research on molecular genetics yields new insights into gene rearrangements and altered gene products, the possibility of molecularly based therapies becomes more intriguing. Future strategies for curing AML may include the transfer of therapeutic genes or genetic engineering to alter the biologic behavior of critical cells.

7. CONTROVERSIAL ISSUES

Controversies exist concerning (1) the intensity and number of courses during induction, consolidation, and intensification treatment; (2) whether all or only high-risk patients should receive matched related allo-SCT in first CR; (3) whether maintenance treatment is necessary and, if so, for how long and in what form; (4) the need for cranial irradiation in CNS-directed treatment; and (5) the general acceptance of risk stratification and its definition.

7.1. How Many Blocks of Intensive Chemotherapy are Mandatory in Children with AML?

Figure 3 presents an overview of five of the most successful pediatric AML treatment protocols. Although there are many differences among the individual regimens, outcomes are quite similar. In all four studies, four to six intensive chemotherapy blocks are given, including induction, consolidation, and intensification. All include ara-C, anthracyclines, and other drugs such as etoposide and 6-thioguanine with known efficacy in AML treatment. For ara-C and anthracyclines, the doses are important, not only the plasma concentrations achieved, but also the cumulative doses, especially with regard to anthracyclines. The schedules of drug administration differ widely. The MRC trial is highly intensive, with different anthracyclines and anthracycline analogs (daunorubicin, mitoxantrone, amsacrine), resulting in a cumulative dosage of about 650 mg/m^2 for the anthracyclines. [An approximately equivalent dose of daunorubicin was calculated by the dose relation of 1:5 (idarubicin or mitoxantrone vs daunorubicin), based on the reports from the AML Collaborative Group and from Thomas and Archimbaud *(25,137)*.] The NOPHO study applies high-dose ara-C in four postremission therapy blocks, whereas the AML-BFM-93 study employs both: high initial plasma levels of anthracyclines followed by high-dose ara-C twice, in an attempt to avoid a high cumulative anthracycline dosage. In the CCG study, the intensive timing of two 4-d blocks during induction/consolidation has produced a major improvement in results compared with outcomes with the standard timing.

This analysis indicates that different methods can be used to achieve similar results (Table 1). To achieve a stable remission, it is important to administer an intensive bone marrow supressive therapy (including high plasma levels of ara-C and anthra-

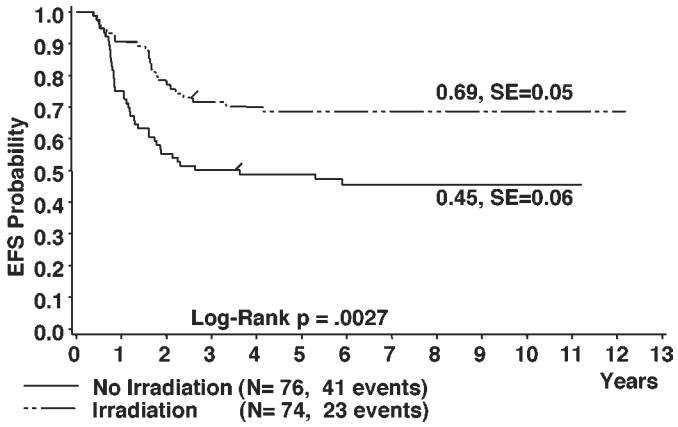

Fig. 4. Estimated probability of event-free survival (EFS ± SE) among patients of study AML-BFM-87, treated with or without cranial irradiation. Only patients without initial CNS involvement and white blood counts <70,000/mm^3 who stayed alive for more than 6 mo were eligible.

cyclines) over 4–5 mo. Furthermore, ancillary aspects, such as the necessity of a highly skilled and experienced medical staff familiar with the specific therapy regimens and the handling of acute and long-term toxicity, need to be considered.

7.2. Do All Patients with AML Qualify for Matched Related allo-SCT in First CR?

Allo-SCT is an effective but highly toxic antileukemic therapy. Some comparable nonrandomized studies demonstrate a survival advantage for patients undergoing allo-SCT; however, comparisons with conventional therapy are biased by patient selection methods *(124)*.

Recently Woods et al. *(125)* reported on their comparison of allo-BMT, auto-BMT, and aggressive chemotherapy in children with AML in remission in the CCG study 2891.

They found that survival was significantly superior in the allo-BMT group compared with auto-BMT and chemotherapy only *(125)*. However, their conclusion that allo-BMT remains the treatment of choice for children and adolescents with AML in remission, when a matched-related donor is available, cannot be accepted without any comment *(126)*. It should be emphasized that >50% of pediatric patients achieving remission will be cured by chemotherapy alone. Survival rates may even increase up to 70% in "good-risk" or standard-risk patients (Table 2). Patients in this risk group will either never relapse or will predominantly experience late relapses. According to our results, the probability of achieving a second remission, especially after a late relapse, is high *(127)*. Thus, given the cure rate with conventional treatment, it can be assumed that although allo-SCT may be less effective after relapse, the overall survival of young patients undergoing this procedure after first relapse would be the same. Therefore, a risk factor analysis is necessary to identify patients in whom allo-SCT in first CR is mandatory. Hence, patients cured by chemotherapy alone would be spared the potential morbidity of transplant procedures.

7.3. Is Maintenance Therapy Necessary? If so, Which Mode and What Duration of Therapy Are Required?

As in ALL, maintenance chemotherapy has been employed in childhood AML to prolong the duration of remission; however, the impact of this treatment phase on cure is not as clear as in ALL. In two recent adult studies, prolonged maintenance regimens applied after four consolidation courses *(128)* or a single highly intensive consolidation course *(129)* did not contribute to long-term results. On the other hand, in a metaanalysis performed by Büchner et al. *(129)*, adult patients treated with standard or intensive maintenance regimens showed a 5-yr continuous CR rate of 25%, compared with only 13% in patients assigned to a reduced dose or no maintenance. These results seem to indicate that, depending on the preceding treatment, maintenance therapy can contribute to an increased long-term survival *(130)*.

In the study AML-BFM 87, the duration of maintenance was reduced from 2 yr in study AML-BFM 83 to 1.5 yr in the following studies, without an increased relapse rate after cessation of therapy. Results of the study CCG-213 *(33)* indicate that maintenance therapy may not be necessary after induction and a postremission intensification phase with high-dose ara-C on an aggressive schedule (every 7 d). However, toxicity and mortality rates were high in this study, and maintenance seemed to benefit those patients who had received the less aggressive ara-C intensification schedules *(131)*. These data support the strategy of an intensive treatment during induction, followed by consolidation and intensification with at least some months of maintenance therapy. Further clinical trials with different durations of maintenance therapy are necessary to support these results. In addition, monitoring of residual disease after intensification may be helpful in making decisions about the cessation of therapy.

7.4. Which Types and Doses of CNS-Related Therapy are Mandatory?

So far, there are no convincing data as to whether inthrathecal and high-dose ara-C or methotrexate alone are sufficient to control MRD in the CNS or whether the addition of cranial irradiation is mandatory. In ALL, high-dose methotrexate together with intrathecal methotrexate can control subclinical CNS disease. Next to the anthracyclines, ara-C is the most effective drug in single-agent treatment of AML *(132)*. In addition, ara-C shows a better drug-resistance profile than methotrexate *(89)*. High-dose ara-C was introduced to increase overall outcome and to control the minimal residual blast cell growth in the CNS. However, in study AML-BFM 87, results in nonirradiated patients treated with high-dose ara-C were inferior compared with those patients treated with high-dose ara-C and cranial irradiation. The cumulative incidence of relapse was 0.53 ± 0.06 in nonirradiated patients compared with 0.29 ± 0.05 in irradiated patients (p = 0.008; see also Fig. 4). This outcome reflects the total number of relapses without CNS involvement (31 of 76 in nonirradiated patients vs 4 of 74 in irradated patients) and only partly the isolated and combined CNS relapses (9 vs 4), thus indicating that residual blasts in the CNS may escape systemic chemotherapy and lead to a recurrence of the initial disease, not only in the CNS but

also in the bone marrow *(update of 28)*. These results and our findings of an unexpected occurrence of blasts in the CSF during consolidation or even after intensification with high-dose ara-C in initially CNS-negative patients indicate that at least for some patients, CNS-directed high-dose chemotherapy or intrathecal therapy is not sufficient. So far, there are no data defining those patients who are most likely to benefit from cranial irradiation, except those with an initial CNS involvement, which is more frequent in children younger than 2 yr of age [our own unpublished observation and Pui et al. *(133)*]. Finally, it is difficult to analyze MRD in the CNS; efforts to resolve this issue are under way.

The question of whether or not cranial irradiation is necessary and, if so, the dose required for optimal results, can only be addressed in clinical trials. In the ongoing trial AML-BFM 98, the equivalence of 12 vs 18 Gy is being tested by randomization. Overall, the number of CNS relapses is low in AML studies, with or without cranial irradiation (but including intrathecal therapy) and is similar to that of the BFM studies without consideration of the possible influence of CNS-directed therapy on the rate of bone marrow relapses.

In different studies the intensity of each therapy cycle is slightly different in terms of the cumulative doses of anthracyclines, ara-C, or CNS-directed therapy or the indications for SCT. Thus, one aim should be to determine the combination of drugs and irradiation dose resulting in minimal side effects. Relevant neurologic sequelae of cranial irradiation can be seen especially in young children *(134)*; however, fewer young children develop AML compared with ALL (median age 8–10 yr vs 4–5 yr, respectively). A recent retrospective analysis of neuropsychological functioning in irradiated compared with nonirradiated patients enrolled in study AML-BFM 87 showed no significant intellectual impairments and only small concentration deficits, especially in girls and children 5 yr or younger at diagnosis *(135)*. Long-term side effects must also be considered after treatment with SCT, which generally produces more severe toxicity than chemotherapy alone *(136)* or following cranial irradiation. One major aim should be to identify the subgroups that benefit most from cranial irradiation and restrict it to these patients.

7.5. Are Risk Groups Required to Stratify Patients for Assignment of Treatment Options?

Although the development of AML therapy during the last 20 yr has resulted in a much better outcome in most children, the prognosis for this disease is much less favorable than for other pediatric malignancies. Current results show that more intensive therapy, although capable of improving outcome, increases rates of therapy-related mortality and morbidity, which must be counteracted by better handling of side effects and improved supportive care. This goal has been met by French pediatric oncology centers, where BMT-related mortality has been reduced from 36 to 3% *(40)*.

In the AML-BFM studies, treatment-related mortality (excluding early deaths due to hemorrhage and leukostasis) does not exceed 4%. This rate remained stable over 20 yr, despite increases in the intensity of chemotherapy, reflecting the influence of more experienced investigators, and the instigation of better supportive care to prevent severe infections during long-term aplasia.

In conclusion, the definition of risk groups for stratified therapy options would allow more intensive therapy to be administered to high-risk patients, as well as the introduction of new drugs (and their inevitably severe side effects) without jeopardizing standard-risk patients. This approach was used in study AML-BFM 93: one cycle of high dose ara-C/mitoxantrone was introduced for high-risk patients only. After achieving significantly better results compared with the previous study, the German investigators applied this strategy to all patients in the current protocol AML-BFM-98, excluding children with Down's syndrome and the M3-FAB subtype.

Although much remains to be learned about the dysregulation and proliferation of the malignant myeloid clones, we can expect that future therapies will be more individualized and less toxic. This advance, coupled with better knowledge of the risk profile of the individual patient, may secure effective treatment for all children with AML.

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18 Treatment of Childhood Acute Myeloid Leukemia

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CONTENTS

1. INTRODUCTION

Acute leukemia is the most common pediatric malignancy. Acute myelogenous leukemia (AML) accounts for 20% of the cases of acute leukemia among children but causes a disproportionate amount of the leukemia-related mortality. Recent progress in the treatment of childhood AML has resulted in a remission induction rate approaching 80%, but long-term event-free survival (EFS) remains between 35 and 50%. This discrepancy suggests that the major barrier to cure is insufficient postremission therapy. Randomized trials have demonstrated improved survival for patients undergoing allogeneic stem cell transplantation during remission, but at the expense of increased treatment-related morbidity and mortality. As a result, the optimal treatment of a patient in remission is not clear.

This chapter highlights the distinctions among different subtypes of AML, discusses treatment strategies for remission induction (as well as postremission therapy), and examines future directions that might eventually lead to improved outcomes for children with AML. In addition, we comment on several controversies relating to pediatric AML therapy.

2. DIAGNOSIS

Most of the symptoms of newly diagnosed AML are related to infiltration of bone marrow with leukemic blasts. The replacement of normal hematopoietic elements with malignant cells results in pancytopenia, which leads to bleeding, symptomatic anemia, and an increased infection risk. Bone pain, presumably from pressure caused by rapidly proliferating leukemic cells in the marrow space, is also seen. AML can infiltrate extramedullary locations, such as the liver or spleen, causing organomegaly in more than half of patients. Central nervous system (CNS) involvement is relatively rare, occurring in only 5–10% of pediatric AML patients, and may be asymptomatic. Finally, AML often presents with a very high white blood cell count, which puts patients at risk for the consequences of hyperleukocytosis and increased blood viscosity. Symptoms of hyperleukocytosis may include respiratory distress, from sludging in the pulmonary vasculature, or altered mental status resulting from CNS hypoxia.

Certain subtypes of AML can cause specific signs and symptoms. For example, monocytic leukemias are associated with infiltration of the skin and gums, as well as the development of chloromas, masses of leukemia cells that can cause symptoms related to their location. Identification of chloromas is critical for overall disease control, as blasts from chloromas have the potential to repopulate the bone marrow and lead to hematologic relapse. Additionally, acute promyelocytic leukemia (APL) is commonly associated with disseminated intravascular coagulation (DIC), with an attendant risk of major hemorrhagic or thrombotic events.

In adults, AML frequently arises in the context of a preexisting myelodysplastic syndrome (MDS) characterized by dysplasia in multiple hematopoietic lineages. This is far less common in children, occurring in <10% of cases. In contrast, *de novo* AML is the norm in childhood.

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There are several ways to classify AML. The most accepted morphologic classification system is the FAB (French–American–British) system, introduced in 1976 and revised in 1985. There are 10 different morphologic categories in the FAB system, and assignment to a particular class is based on morphology, immunohistochemistry, and expression of cell surface antigens. In addition to FAB class, AML is also characterized by cytogenetics. A host of recurrent translocations have been identified; these have yielded important clues the molecular events underlying leukemogenesis and may have prognostic significance as well.

3. THERAPY

3.1. General Aspects of Therapy

The initial goal of therapy is the induction of a remission. This usually involves the administration of intensive chemotherapy, with its associated morbidity and mortality. Cytotoxic therapy for AML always results in transient but severe myelosuppression with a risk of bleeding and infection. Only after normal bone marrow cellularity is restored can remission status be accurately assessed. Upon recovery of normal hematopoietic function, postinduction therapy is administered. This can consist of relatively intensive chemotherapy alone or myeloablative therapy with either autologous or allogeneic stem cell support. Some treatment regimens include prolonged periods of maintenance chemotherapy, analogous to standard care for patients with acute lymphoblastic leukemia (ALL), whereas other regimens do not.

An important ancillary issue is CNS prophylaxis. In pediatric ALL patients, this is a critical component of therapy, since CNS involvement at diagnosis is common and with inadequate prophylaxis, CNS relapse rates approach 50%. CNS involvement in AML is less common, but is still a significant enough problem that prophylaxis is mandatory. The relative importance of cranial radiation, systemic therapy that crosses the blood-brain barrier, and intrathecal therapy remain to be determined.

Finally, with a few exceptions to be discussed in detail below, all AML patients are treated the same. With increasing knowledge of the biologic diversity of AML, and the differing prognoses associated with different biologically defined subclasses, the era of treating all AML patients in the same manner will be replaced by an era of risk-directed and biologically based therapies. This approach is already evident in the treatment of AML patients with Down's syndrome and patients with APL.

The monitoring of residual disease is an important component of therapy. Morphologic methods are relatively insensitive, although they remain the gold standard for determining remission status. More sensitive methods for determining the presence of minimal residual disease, such as immunophenotyping, karyotyping, and polymerase chain reaction (PCR), are being introduced, but the clinical significance of minimal numbers of leukemic cells identified with these techniques for the most part remains unclear.

3.2. Remission Induction Therapy

The primary goal of remission induction therapy is the achievement of a complete remission (CR). A secondary goal is to provide the foundation for curative therapy. Thus, even though regimens have been developed that allow the vast majority of patients to achieve a remission, further refinements have been aimed at improving long-term outcome as well. A common thread among all the major international cooperative groups has been the intensification of induction therapy, either through dose intensification or through time intensification.

The standard remission induction regimen for many years consisted of a 7-d infusion of cytarabine (ara-C) along with three doses of an anthracycline, usually daunorubicin. When accompanied by 7 d of 6-thioguanine, this regimen is abbreviated DAT. The U.S. Pediatric Oncology Group (POG) pursued dose intensification of ara-C, substituting 6 doses of high-dose ara-C (HDAC; 3 g/m^2/dose) for a second course of DAT during induction therapy on study 8498 *(1)*. Although there was no significant difference in the remission induction rate (85% in both patient groups), the patients treated with HDAC had an improved 3-yr EFS (34% vs 29%) and disease-free survival (42% vs 34%). Although these results do not demonstrate an improvement in remission induction with HDAC, there is a suggestion that HDAC during induction might improve long-term results.

The U.K. Medical Research Council (MRC) directly tested the importance of dose intensification in their AML-9 trial *(2)*. In this study, patients were randomized to receive either DAT 1+5 (daunorubicin for 1 d, ara-C and 6-TG for 5 d) or DAT 3+10 (the identical drugs but for 3 and 10 d, respectively). Although the patients treated with 3+10 experienced more toxic deaths (21% vs 16%), there was a higher remission induction rate (66% vs. 61%) and shorter time to CR (34 vs 46 d). These numbers are statistically significant and support the concept that intensive therapy improves remission induction rates. Remission induction rates were further improved in the AML-10 trial *(3)*. On that trial, patients were randomized between two different induction regimens: two cycles of DAT or DAE (daunorubicin, ara-C, etoposide), followed by a course of amsacrine, ara-C, and etoposide, as well as a course of mitoxantrone and ara-C. After all four cycles, the remission induction rate was 92% and was 83% after just two cycles.

The German Berlin–Frankfurt–Münster (BFM) group has also confirmed the importance of intensive therapy for remission induction. Their AML-BFM-78 trial consisted of an 8-wk remission induction regimen followed by 2 yr of maintenance therapy *(4)*. This regimen resulted in a complete remission for 80% of patients and long-term EFS for 38%. The subsequent study, AML-BFM-83, added an 8-d course of DAE to the induction regimen from AML-BFM-78 *(4)*. Although this did not significantly improve the rate of remission induction, there was a significant improvement in long-term EFS (49% vs 38%).

The U.S. Children's Cancer Group (CCG), unlike the other major cooperative groups, pursued intensification of timing. This group performed a randomized study, CCG-2891, that directly compared identical remission induction regimens, two cycles of dexamethasone, cytarabine, 6-thioguanine, etoposide, and rubomycin (DCTER), the second cycle given either after hematopoietic recovery from the first, or after 6 d of rest *(5)*. Although there was no significant difference in remission induction rate, the 3-yr EFS rate was 42% for the intensive

timing group, compared with 27% for the standard timing arm. This survival advantage continues even at 8 yr of follow-up *(6)*.

3.3. Postinduction Therapy

After a CR is obtained, further chemotherapy is still required to achieve a cure. There is no consensus regarding the optimal number or intensity of cycles of postinduction chemotherapy. The POG protocol 9822 utilized three highly intensive cycles of chemotherapy followed by myeloablative therapy and autologous peripheral blood stem cell rescue (unpublished data). The MRC, in their AML 10 trial, treated patients with two highly intensive cycles of chemotherapy *(3)*. The Nordic Society of Pediatric Hematology and Oncology AML-88 trial contained four intensive cycles of chemotherapy *(7)*. In contrast to these regimens, the BFM trials utilize two cycles of intensive chemotherapy followed by a 2-yr low-intensity maintenance regimen *(4)*. The AML-BFM-93 trials randomized patients to receive the HAM regimen (high-dose ara-C and mitoxantrone) either as a second or third course of therapy and found no significant difference in outcome *(8)*.

The CCG conducted a randomized trial of differing postinduction therapies. Patients enrolled in CCG-213 who achieved a complete remission were randomized to an intensive chemotherapy regimen containing ara-C and L-asparaginase, or to a less intensive regimen followed by prolonged maintenance therapy *(9)*. The children treated with the more intensive postinduction regimen had a 5-yr survival rate (from the end of consolidation) of 68%, compared with 44% for the less intensive arm. Although this study does not demonstrate that this particular postinduction regimen is optimal, it does argue strongly that more intensive therapy of a short duration provides a superior outcome compared with less intensive therapy with a prolonged maintenance regimen.

3.4. Stem Cell Transplantation

Stem cell transplantation plays a dual role in the treatment of AML. Because the dose-limiting toxicity of the effective chemotherapy agents is myelosuppression, stem cell support allows this limit to be exceeded. Additionally, infusion of allogeneic stem cells offers an immune-based graft-versus-leukemia (GvL) effect. Autologous stem cells allow the dose-limiting toxicity to be exceeded and carry no risk of graft-versus-host disease (GvHD) but provide no GvL effect and have the potential to be contaminated with leukemic cells. Allogeneic stem cells from an HLA-identical family member provide all the benefits mentioned, but availability is limited. Stem cells from an unrelated donor are often available in the absence of a family donor, but they carry an increased risk of GvHD.

Multiple studies have demonstrated the efficacy of an allogeneic stem cell transplant from an HLA-identical sibling donor for children with *de novo* AML in first remission. These studies have used a biologic randomization strategy, that is, patients with an identified HLA-matched sibling donor were offered bone marrow transplantation (BMT), whereas patients without such a donor were randomized to receive an autologous transplant or intensive chemotherapy alone. POG 8821 followed this approach *(10)*. Patients who were treated with allogeneic BMT had a 3-yr event-free survival of 52%, compared with 37% for patients treated with either chemotherapy alone or autologous BMT. The CCG reported similar findings from the CCG-2891 study *(6)*. When analyzed by intention to treat, patients on this study who received allogeneic BMT had a 60% survival rate, compared with 48% for patients treated with autologous transplants and 53% for patients treated with chemotherapy alone. Interestingly, the patients treated on the intensively timed remission induction arm had even better results (70% for patients treated with allogeneic BMT, compared with 54% for patients treated with autologous BMT and 57% for patients treated with chemotherapy alone). The European Organization for the Research and Treatment of Cancer (EORTC) reported a similar experience in their AML 8A trial, with long-term disease-free survival from the time of CR of 46% for allogeneic transplant recipients but only 33% for other patients *(11)*.

Interestingly, the results of the MRC AML-10 protocol were different. This study failed to support a survival benefit for BMT, although there was a trend toward improved outcome for allogeneic transplant recipients *(3)*. Children enrolled on this trial were biologically randomized to allogeneic BMT or either autologous BMT or chemotherapy alone. The long-term survival rate from CR induction was 70% for children who received an allogeneic transplant, compared with 60% for children without a donor, but this was not a statistically significant difference. Event-free survival was also statistically similar between the two groups (61% for transplant recipients compared with 50% for patients without a donor).

In contrast to the case for allogeneic BMT, there is very little evidence that autologous transplantation improves outcome. The CCG reported 48% long-term survival for patients receiving autologous transplantation, compared with 53% for patients treated with chemotherapy alone on the CCG-2891 study *(6)*. The POG 8821 and MRC AML-10 trials also both demonstrated equivalency between intensive chemotherapy and intensive chemotherapy with autologous stem cell support *(3,10)*. Although AML-10 yielded an improved 7-yr relapse-free survival rate for patients treated with autologous transplantation compared with chemotherapy alone (69% vs 48%), there was no difference in overall survival, in part because patients who were transplanted fared significantly worse after a relapse (7% survival vs 26%), and autologous transplantation was associated with a significantly higher rate of toxic death (9% vs. 1%). Similar results were obtained by another European study, AIEOP/LAM 87. Using a biologic randomization strategy, this group reported 51% long-term disease-free survival for patients undergoing allogeneic BMT, which was significantly better than the 21% long-term disease-free survival of patients treated with autologous BMT and the 27% survival of patients treated with chemotherapy alone *(12)*.

A third transplantation option for AML patients is the use of alternative, usually unrelated donors. This is an effective approach, but it carries a significant risk of treatment-associated morbidity and mortality. A recently published update of the Seattle experience with 161 AML patients ranging in age from 1 to 55 yr, reported a 5-yr leukemia-free survival rate of 50% for patients transplanted in CR1, and 28% for patients transplanted in CR2 *(13)*. The Italian Bone Marrow Transplant Group reported a 31% 3-yr disease-free survival for

AML patients, but at the price of a 44% transplant-related mortality rate for patients transplanted since 1993 *(14)*. A similar 2-yr disease-free survival rate for children with AML was reported from the University of Minnesota, but with a rate of severe acute GvHD of 23% and a 50% incidence of chronic GvHD *(15)*. Interestingly, the European Group for Blood and Marrow Transplantation (EBMT) reported equivalent leukemia-free survival for AML patients treated with autologous transplantation or bone marrow from an unrelated donor but superior overall survival in recipients of an autologous graft (69% vs. 42%) among patients with AML in CR1 *(16)*. These data suggest that, although unrelated donor transplants are effective, they carry significant risks of procedure-related morbidity and mortality and therefore should be reserved for patients with very high-risk disease that portends a very low chance of survival.

3.5. CNS Prophylaxis

In contrast to treatment strategies in ALL, routine cranial irradiation is not a general component of therapy for AML. Dahl et al. *(17)* reported that cranial radiation impacts the rate of CNS relapse but not overall survival. The BFM study AML-87 tested prospectively whether cranial irradiation, which was a routine component of prior BFM regimens, could be replaced by late intensification with high-dose ara-C *(18)*. Interestingly, patients who received cranial radiation had a superior probability of relapse-free interval of 5 yr compared to patients who were not irradiated (0.78 and 0.41, respectively). Of note, randomization was stopped early in this study, and most of these data are based on nonrandomized patients. It therefore remains to be determined whether cranial radiation indeed protects against bone marrow relapse.

3.6. Prognostic Factors and Risk-Adapted Treatment Strategies

Every major cooperative group has attempted to identify criteria that distinguish different risk categories among pediatric AML patients, analogous to the criteria used to classify ALL patients as low, standard, or high risk. Earlier studies attempted to utilize relatively straightforward clinical criteria, such as FAB subtype, white blood cell count at diagnosis, and age, but independent prognostic variables proved elusive. More recently, the BFM has examined the prognostic significance of cytogenetic abnormalities and immunophenotype *(19)*. Although expression of the cell surface antigen CDw65 was associated with an increased CR rate, there was no effect on EFS. In contrast, t(8;21) and inv(16) were associated with superior EFS compared with other karyotypes (57% vs 32%). Interestingly, this was explained almost completely by an improved initial response rate, because the event-free interval after CR induction was not affected by cytogenetics. Other variables that have been associated with a poor outcome in that study were an elevated leukocyte count, age <2 yr, FAB subtypes M4, M5, M6, and M7, and a d-15 bone marrow aspirate that contained >5% residual blasts.

The MRC has also derived a series of criteria for risk stratification, based on a retrospective analysis of the results of their AML-10 trial *(20)*. The criteria include response to induction therapy, cytogenetics, and FAB subtype. The good-risk group consists of patients with a favorable karyotype [t(8;21), t(15;17), inv(16)] or FAB M3 blast cells without t(15;17). The poor-risk group consists of patients with an unfavorable cytogenetic abnormality [monosomy 5 or 7, del(5q), abn(3q), or a complex karyotype] or with persistent disease after the first course of chemotherapy. The standard-risk group contains all other patients. The prognostic value of these groupings is being tested prospectively in the AML-12 trial.

The POG, in a retrospective analysis of trial 8821, also identified three risk groups *(21)*. The high-risk patients were those with a chromosomal abnormality other than t(8;21) or inv(16). The intermediate-risk group consisted of male patients with t(8;21), inv(16), or normal chromosomes, and the low-risk group was female patients with those same chromosomes.

More recently, internal tandem duplication (ITD) of the *FLT3* gene was identified as an adverse prognostic factor among children treated on CCG-2891. *FLT3* encodes a receptor tyrosine kinase, and several adult studies demonstrated that the presence of an internal tandem duplication of the juxtamembrane region of the cytoplasmic domain of the receptor is associated with poor outcome *(21,23)*. A review of the results of CCG-2891 revealed that patients with *FLT3*/ITD-positive leukemia had an 8-yr EFS rate of 7%, compared with 44% for those without the ITD *(24)*. Multivariate analysis demonstrated that the presence of *FLT3*/ITD was the most significant prognostic factor for a poor outcome. Most other studies have confirmed the adverse prognostic significance of *FLT3*/ITD mutations, although there is some evidence that intensification of therapy with transplantation may in part overcome the impact of this prognostic factor.

3.7. Therapy for Specific Patient Groups

3.7.1. Down's Syndrome

Children with Down's syndrome have a 20-fold increased incidence of leukemia compared with other children *(25)*. In this population, unlike in unaffected children, myeloid leukemia frequently develops from an MDS, and there is an increased incidence of M7 AML *(26)*. Interestingly, although this FAB subtype is often quite difficult to treat in patients without Down's syndrome, those with the syndrome respond unusually well to therapy *(27,28)*. This good response to chemotherapy is a characteristic of the patients rather than being specific for M7 AML and has been attributed to enhanced intracellular conversion of ara-C to ara-CTP, the active form of the drug *(29)*. Given the increased sensitivity to ara-C, AML in these patients represents a unique situation and should probably be treated with a regimen that contains high-dose ara-C. Because of the increased cure rate, allogeneic BMT in CR1 is not recommended for these patients.

Patients with Down's syndrome are also prone to the development of a transient myeloproliferative disorder (TMD) in infancy *(30)*. As implied by its name, this is a transient disorder that spontaneously resolves with no specific intervention. Trisomy 21 appears to be necessary for the development of TMD, but there are reports of the trisomy being limited to the clonal cells, suggesting that it may develop in infants without Down's syndrome *(31)*. Children with TMD require close follow-up, since a significant proportion develop AML *(30)*.

3.7.2. Acute Promyelocytic Leukemia

The treatment of APML (FAB M3) is the best developed example of the use of differentiation therapy in the treatment of cancer. APML blasts are characterized by chromosomal translocations, such as t(15;17), involving one of the retinoic acid receptors, *RARα*. The resulting disruption of the receptor is thought to interfere with the normal differentiation pattern directed by retinoic acid, resulting in leukemia *(32)*. All-*trans*-retinoic acid (ATRA) binds to the chimeric *RARα* and overcomes the differentiation block. The combination of ATRA and cytotoxic therapy has transformed APML from a disease with a dismal prognosis to one that is highly favorable *(33)*.

APL is also amenable to treatment with another noncytotoxic agent, arsenic trioxide. At low doses, arsenic causes the differentiation of APL blasts, and at higher doses it induces apoptosis *(34)*. Sensitivity to arsenic seems to be independent of sensitivity to ATRA, suggesting that these agents work by different mechanisms. Ongoing clinical trials are aimed at identifying the optimal combination of ATRA, arsenic, and cytotoxic drugs for the treatment of APML and are also investigating the possibility of using arsenic as a differentiation agent for other subtypes of AML.

3.7.3. Core-Binding Factor Leukemias

Among the many recurrent chromosomal translocations identified in patients with AML, two, [t(8;21) and inv(16)] involve transcription factors that are members of the core-binding factor family *(35)*. Several studies of AML in adults have identified these translocations as favorable prognostic features, although whether this holds for pediatric AML remains to be seen. Bloomfield et al. *(36)* demonstrated that AML patients with these karyotypes are particularly sensitive to ara-C. Patients were randomized to standard-, intermediate-, or high-dose ara-C intensification, and long-term remission rate was compared with karyotype. The CR rate at 5 yr among patients with core-binding factor leukemias was 50%, compared with 32% for patients with normal karyotypes and 15% for patients with other abnormalities. These differences were statistically significant.

4. TOXICITY AND SUPPORTIVE CARE

The principal causes of death among patients with AML are hemorrhage, infection, and the consequences of leukostasis. These can be caused by the primary disease or are seen as toxicities of the chemotherapy. Each is amenable to treatment, and appropriate supportive care is therefore critical for patient survival.

4.1. Hemorrhage

Hemorrhage is often the result of profound thrombocytopenia, which can be a consequence of marrow infiltration with leukemic blasts, dysplastic hematopoiesis, or chemotherapy-related myelosuppression. Platelet transfusions to correct the thrombocytopenia are safe, even in the setting of profound hyperleukocytosis. Hemorrhage can also develop as a consequence of leukostasis. In patients with M7 AML, bleeding can occur even with a normal platelet count, because the platelets are often dysfunctional, having developed from abnormal megakaryocytes.

Another cause of bleeding in an AML patient is DIC, which is often seen in patients with APML, particularly at presentation. The leukemic blasts contain granules with proteolytic enzymes, and the release of these enzymes, either spontaneously or as a result of the institution of cytotoxic therapy, can trigger DIC. The risk of hemorrhagic death in APL patients was decreased in patients treated with ATRA in addition to cytotoxic drugs in one study *(37)*, and this treatment decreased the severity of hemorrhagic complications in another *(38)*.

4.2. Infection

Severe infections complicate all phases of AML therapy *(38)*. At the time of presentation, patients may have pancytopenia, with the attendant risk of infection associated with neutropenia. Even patients with a normal or elevated white blood cell count are at risk for infection, because the neutrophils that are present may not be functional. Cytotoxic therapy increases the risk of fatal infection by several interacting mechanisms. In addition to inducing prolonged and severe neutropenia, cytotoxic drugs cause significant mucosal injury. This effect reduces the barrier function of the mucosa, allowing access of intestinal flora and skin flora to the bloodstream. Finally, since the classical signs of infection, such as erythema, pain, and formation of pus, all require inflammatory cytokines produced by neutrophils, the presentation of even severe infections can be quite subtle, potentially delaying the institution of aggressive antimicrobial therapy. Antimicrobial prophylaxis, particularly intestinal sterilization with nonabsorbable antibiotics and antifungal agents, and the prompt institution of broad-spectrum antibiotic treatment are essential supportive measures for AML patients.

4.3. Leukostasis

Leukostasis refers to the consequences of the hyperviscosity associated with an extremely elevated white blood cell count (>100,000/μL). Clinical symptoms of leukostasis include neurologic symptoms (ranging from headache through confusion, sedation, and coma) and respiratory distress *(40)*. These symptoms are particularly common among patients with M4 or M5 AML. Effective treatment of leukostasis requires a prompt decrease in the white blood cell count. This can be accomplished through leukopheresis, exchange transfusion, and the administration of chemotherapy. Leukapheresis requires the placement of central catheters of sufficient caliber to support very high flow rates. For technical reasons, this may not be an option for very small children. Exchange transfusion, either with packed red blood cells or with fresh frozen plasma, can be performed safely even in very small children and may be associated with fewer complications, such as volume shifts and metabolic imbalances. Exchange transfusion with packed red blood cells has the further advantage of treating severe anemia without the increased blood viscosity that would accompany a simple transfusion.

The prompt administration of cytotoxic therapy is also critical for the treatment of leukostasis, since blasts removed from the circulation are rapidly replaced by blasts from the bone marrow. Hydroxyurea can be useful in slowing the proliferation of leukemic blasts, but this too is only of temporary benefit. The administration of induction chemotherapy to a

patient with hyperleukocytosis is often accompanied by the metabolic derangement associated with acute tumor lysis syndrome (hyperkalemia, hyperphosphatemia, and hyperuricemia). These abnormalities must be aggressively managed with hydration, urine alkalinization, and administration of allopurinol. In some cases, hemodialysis is necessary to control these metabolic disturbances, particularly in the setting of preexisting renal insufficiency.

5. FUTURE DIRECTIONS OF AML THERAPY

Current treatment regimens for childhood AML are quite good at inducing remission. Nevertheless, cure rates remain unacceptably low. AML therapy is quite toxic, so future improvements in outcome are unlikely to result from simple dose intensification. Until new therapeutic modalities are developed, optimized use of currently available agents will be the major source of improvements in patient care.

5.1. Optimizing the Use of Currently Available Cytotoxic Drugs

There are several variables that can be altered in an attempt to optimize the efficacy of chemotherapeutic agents, including the timing of delivery, the dose of the drug, and the combinations of drug that are administered. Relatively new treatment regimens have been developed that address each of these variables.

5.1.1. High-Dose Cytarabine

Because ara-C is only active against cells in the S-phase of the cell cycle, it is usually administered as a continuous infusion over 24–96 h in an attempt to maximize exposure to cycling cells. The sensitivity of leukemic blasts to ara-C is in part dependent on the intracellular concentration of the drug, which is converted to ara-dCTP, the active form of the compound. Impaired drug import is a major mechanism of resistance, and this can be overcome by using doses a log or higher than conventional dosing *(41)*. HDAC is often effective for patients who are refractory to standard doses. Both the Southwest Oncology Group and the Australian Leukemia Study Group have shown an improvement in relapse-free survival for patients treated with HDAC during induction, compared with patients treated with standard doses *(42,43)*. Subsequently, HDAC has been incorporated into most standard AML regimens.

5.1.2. Timed Sequential Therapy

The rationale for timed sequential therapy emerged from the work of Burke and colleagues in the early 1970s. They were able to show that an initial dose of chemotherapy recruited otherwise quiescent leukemic blasts into the cell cycle and that administration of a second dose of chemotherapy coincident with the peak recruitment of cells into the cycle dramatically improved the rate of remission induction *(44)*. The CCG conducted a randomized prospective trial to compare identical chemotherapy regimens delivered with standard timing or as timed sequential therapy *(5)*. Although there was no difference in remission induction, there was a significant improvement in the 3-yr EFS rate (42% for the intensive timing group, compared with 27% for the standard timing group). The survival advantage remained at the most recently published follow-up, at a median of 8 yr *(6)*. The survival advantage for patients treated with intensive timing is independent of postremission therapy. However, a definitive test of timed sequential therapy vs dose intensification would compare the same amount of chemotherapy given over the same period either in a timed sequential regimen or as a continuous infusion.

5.1.3. Novel Drug Combinations

An interesting novel drug combination is the pairing of fludarabine with ara-C to enhance the activity of the latter agent. As described previously, ara-C must be converted to ara-dCTP to be active. Phosphorylation by deoxycytidine kinase is the rate-limiting step in this process. Gandhi and Plunkett demonstrated that fludarabine increases the activity of deoxycytidine kinase, increasing the accumulation of ara-dCTP in K562 cells *(45)*. A pilot study at M.D. Anderson confirmed that fludarabine potentiates the metabolism of ara-C in patients *(45)*. The CCG incorporated this drug combination into an effective salvage chemotherapy regimen for patients with refractory or relapsed AML *(46)*, and other groups have administered this drug pair with granulocyte colony-stimulating factor (G-CSF) to patients with refractory, relapsed, or poor-prognosis AML with some success *(47,48)*. Whether this combination has a significant impact on long-term outcome is unclear.

5.2. New Drugs

5.2.1. 2-Chlorodeoxyadenosine

2-chlorodeoxyadenosine (2-cdA) has shown some activity in the treatment of adults with recurrent or refractory AML. In one study, 32 of 36 patients cleared their circulating blasts. In a phase II study in children, the overall response rate was 59%, although only 6 of 22 patients (27%) achieved a remission *(49)*. This drug is reasonably well tolerated, with higher doses causing prolonged myelosuppression or peripheral neuropathy.

5.2.2. Gemcitabine

Gemcitabine is a deoxycytidine analog, like ara-C, and requires intracellular phosphorylation for activation. It has greater membrane permeability, enzyme affinity, and more prolonged intracellular retention than ara-C *(50)*. Given the efficacy of ara-C for myeloid leukemias, it is reasonable to expect that gemcitabine will also be an active agent. Clinical trials to address the activity of this agent in children with leukemia are ongoing.

5.2.3. R115777

Unlike the two drugs discussed above, R115777 is not a traditional cytotoxic agent; rather, it is an example of a molecularly targeted drug. R115777 is an inhibitor of farnesylprotein transferase, which catalyzes the posttranslational farnesylation of RAS and other proteins. The RAS family of proteins are guanine nucleotide-binding proteins involved in signal transduction pathways related to proliferation, differentiation, and apoptosis. Isoprenylation of RAS is essential for its function, and R115777 inhibits this process. A phase I trial of this drug in adults with refractory leukemias showed responses in 10 of 34 patients *(51)*. Clinical trials in children are under way.

5.2.4. Imatinib Mesylate (STI-571)

Developed as a specific inhibitor of the BCR-ABL tyrosine kinase oncoprotein in Philadelphia chromosome-positive

chronic myelogenous leukemia (CML) *(52)*, STI-571 also holds promise for the treatment of AML. This compound inhibits the activity of the wild-type c-Kit tyrosine kinase, a commonly expressed kinase in AML. There is in vitro evidence that interfering with the activity of c-Kit might be toxic to leukemic blasts *(52)*, and ongoing trials are assessing the efficacy of this drug in AML patients.

5.2.5. BCL2 Antisense Oligonucleotide

Another example of a molecularly targeted drug is an antisense oligonucleotide directed against the antiapoptotic gene *BCL2*. *BCL2* was first identified in B-cell lymphomas but has since been shown to be a central mediator of resistance to apoptosis in both normal and malignant cells *(53)*. Preclinical data support the concept that treating cells with an antisense oligonucleotide that downregulates *BCL2* makes these cells more sensitive to chemotherapeutic agents *(54)*. The results of a pilot study of this agent in AML patients, recently presented at a meeting sponsored by the American Association for Cancer Research, showed promise and will certainly lead to further testing of this strategy in the near future.

5.3. Differentiation Therapy

The most prominent example of differentiation therapy is the use of ATRA for the treatment of APL. In this disorder, a characteristic chromosomal translocation, most frequently t(15;17), results in the fusion of a portion of a gene known as *PML* with *RARα*. This fusion protein is thought to interfere with the normal differentiation program directed by retinoic acid. ATRA, in pharmacologic doses, overcomes this block of differentiation, allowing the leukemic blasts to undergo terminal differentiation and then apoptosis *(32)*.

Although ATRA is the most developed form of differentiation therapy, other agents with similar activity are being tested. Arsenic trioxide was originally developed as an alternative differentiation agent for APL cells *(34)*. At low doses it does cause these cells to differentiate, whereas at higher doses it induces apoptosis. Ongoing clinical trials will address the utility of arsenic for other subtypes of AML.

Another differentiation-inducing agent in active trials is 5'-azacytidine *(55)*. Like arsenic, 5'-azacytidine is cytotoxic at high doses. Interestingly, at lower doses this drug inhibits DNA methyltransferases. Aberrant methylation of the promoters of tumor suppressor genes, leading to the silencing of their expression, is a frequent event in nearly all human cancers, including AML *(56)*. Ongoing studies are investigating the incorporation of 5'-azacytidine into AML therapy, in the hope that reinducing the expression of tumor suppressor genes might facilitate either the differentiation of the leukemic blasts or their apoptosis.

5.4. Immunotherapy

Immunotherapy for AML takes several forms, including treatment with toxin-conjugated antibodies and tumor vaccines. The most developed immunotherapy is the calicheamicin-conjugated anti-CD33 antibody commercially known as gemtuzumab ozogamicin, (Mylotarg™). CD33 is expressed on the vast majority of AML blasts but is not expressed on normal hematopoietic stem cells or on mature myeloid cells, making this an ideal target. Recently published results demonstrate the efficacy of this drug as a single agent *(57,58)*. This is a relatively well-tolerated treatment, with side effects limited to infusional toxicities such as fever and chills and transient liver toxicity with elevated transaminases, although there have been reports of venoocclusive disease and a risk of prolonged thrombocytopenia. Despite these difficulties, this antibody has significant activity as a single agent and is now being tested in combination with chemotherapy.

A key issue concerning agents targeted to a differentiation antigen such as CD33 is whether or not that antigen is present on the leukemic stem cell. CD33 does not appear to be expressed on the leukemic stem cells of most AML cases *(59)*, with the exception of APL, whose stem cells are probably CD33-positive *(60)*. This could limit the ability of Mylotarg to produce durable remissions or cures, which would require elimination of the leukemic stem cell. Interestingly, a recent study combined ATRA with Mylotarg, without conventional chemotherapy, and demonstrated quite encouraging, albeit preliminary, results for adults with APL *(61)*. It is less clear how such strategies will work in AML subtypes in which the leukemic stem cell is not CD33-positive.

Another approach to immunotherapy for AML is the use of tumor vaccines and the conversion of leukemic blasts into antigen-presenting dendritic cells. There is a growing literature suggesting that leukemic blasts can be treated in culture with cytokines to induce at least partial differentiation into dendritic cells *(62)*. Since dendritic cells are the most efficient antigen-presenting cells, it is quite likely that induction of differentiation in vitro would make these cells highly immunogenic and that this might be the basis for a very effective tumor vaccine. Other approaches to antitumor immunization, including the use of the immunostimulatory cytokine granulocyte-macrophage (GM)-CSF, have been reviewed relatively recently *(63)*. Clinical trials in adults and children have been initiated.

5.5. Monitoring Residual Disease

The formal definition of CR, at least in American clinical trials, is based on bone marrow morphology, with the sole requirement being a cellular marrow with <5% blasts. Molecular technologies, such as cytogenetics, PCR analysis and immunophenotyping, can detect a much lower level of residual disease. This leaves open the question of how significant the detection of minimal residual disease is for a patient's prognosis. It is likely that the clinical significance of minimal residual disease as detected by molecular techniques will vary with the disease. For example, it is clear that the persistence or reappearance of the *PML-RARα* fusion gene in patients with APML is highly predictive of clinical relapse *(64)*. By contrast, the *AML1-ETO* rearrangement generated by the t(8;21) translocation can be seen in normal controls without leukemia and in AML patients who seemingly remain in long-term unmaintained remission *(65)*.

A question related to the significance of molecularly detectable minimal residual disease is the timing of therapy based on the detection of a molecular relapse. Clearly, in the case of patients with t(8;21), salvage therapy should not be instituted upon detection of the fusion gene, which can be detected for years in patients who lack overt disease. In the case of APML, there is evidence that early treatment of a molecular relapse is

advantageous. Lo Coco et al. *(66)* reported results on 14 patients with APL treated with reinduction therapy at the first sign of molecular relapse. The 2-yr survival rate from the time of relapse for these patients was 92%, compared with 44% in a prior series of patients treated the same way but at the time of hematologic relapse. Although this observation is highly suggestive of an improved outcome with early treatment, the sample size is small, and the difference in survival at 2 yr may simply reflect earlier detection of disease in the study group than in the historical controls. Nonetheless, this approach deserves to be tested in a prospective study.

6. CONTROVERSIES

6.1. How Many Cycles of Intensive Chemotherapy Are Required for Children with AML?

The recent history of AML therapy shows that increasing intensity of therapy has improved survival time. Increased treatment intensity has also been accompanied by increased toxicity. Clearly a balance must be struck between therapeutic efficacy and regimen-related morbidity and mortality. No studies have been performed in either adults or children to determine the optimal duration of intensive therapy. Tallman et al. *(67)* examined the impact of postremission chemotherapy before HLA-matched sibling donor BMT on the survival of adult patients with AML. They concluded that there was no survival advantage for patients who received postremission therapy compared with patients who proceeded directly to BMT as soon as a remission was achieved. This would suggest, at least in adult patients who will be receiving an allogeneic transplant, that one or two intensive cycles of chemotherapy are sufficient. This study is flawed, however, in being a retrospective analysis, with no standardized postremission therapy and no randomization to consolidation therapy vs immediate transplantation. It is therefore difficult to draw firm conclusions.

6.2. Do All AML Patients Require an Allogeneic Stem Cell Transplant in First Complete Remission?

Data from both major U.S. cooperative groups would suggest that childhood AML patients benefit from stem cell transplantation in first complete remission. The published data, however, are derived from studies without risk stratification, that is, patients for whom a matched sibling donor was available were offered transplantation, and their outcomes were compared with those of patients who did not have such an option and who were treated with either autologous transplants or intensive chemotherapy alone. Because many patients treated with chemotherapy alone survived their disease, it is clear that an allogeneic transplant is not essential for survival. No study published by an American cooperative group has stratified the decision between transplantation and intensive chemotherapy based on cytogenetic abnormalities that might convey prognostic significance. Perhaps patients with t(8;21), inv(16), or t(15;17) could be treated with intensive chemotherapy with equivalent survival and thus be spared the toxicity of stem cell transplantation.

This question is further complicated by the results of the MRC-10 trial, which did not show a survival advantage for allogeneic transplantation compared with chemotherapy alone *(3)*. There was a smaller risk of relapse after transplantation, but this was offset by an increase in regimen-related mortality. Patients enrolled in MRC-10 were treated with significantly more anthracycline than were patients enrolled on CCG 2861. Perhaps the difference between these studies reflects differences in chemotherapy. Future improvements in supportive care post transplantation might diminish the risks of BMT, making this procedure the treatment of choice regardless of the chemotherapy used as an alternative. Alternatively, improvements in targeted approaches to treatment may in most patients eliminate the advantage of allogeneic stem cell transplantation.

A key issue to be addressed in the decision to perform BMT in CR1 is the degree of benefit of transplantation relative to chemotherapy weighed against the increase in toxicity. Another consideration is the efficacy of salvage chemotherapy and BMT in CR2 for patients who relapse after chemotherapy alone. A randomized study to test this important question has not been performed. Most studies, when analyzed by intent to treat, show a benefit for allogeneic transplantation in first remission. However, retrospective or *post hoc* analysis of some studies, such as those from the MRC, suggest that the deferment of matched family donor allogeneic transplantation until CR2 may be beneficial. In other patients who may have AML with a very poor prognosis, the use of alternative donor allogeneic transplantation is worth testing.

6.3. Is Maintenance Therapy Required?

There is no published evidence supporting the use of maintenance therapy for children with AML. The BFM group still treats patients with a prolonged period of low-intensity maintenance therapy. The AML-BFM-87 study reduced the maintenance period by 6 mo, from 2 yr to 1.5 yr with no change in outcome *(4)*. The CCG randomized patients to receive or not to receive maintenance therapy in the CCG-213 study *(9)*. There was no significant difference in survival between patients who received or did not receive maintenance therapy. In some subgroups, outcome was actually inferior for patients who received maintenance therapy, presumably from an increased rate of infection. The survival rates for patients treated by the CCG and for patients treated by the BFM are quite similar, despite this basic difference in approach, further arguing against the need for maintenance therapy.

6.4. What is the Optimal CNS Prophylaxis and Treatment?

This is another area of AML therapy without substantial data on which to base treatment decisions. It is clear that CNS prophylaxis is necessary, but the relative importance of intrathecal therapy, infusional ara-C, or cranial radiation is not clear. Only the BFM has attempted to address this question directly. In their AML-BFM-87 trial, patients were randomized to receive or not receive cranial radiation. Interestingly, patients who did not receive cranial radiation were not only more likely to relapse in their CNS but were also more likely to suffer a hematologic relapse *(18)*. The BFM concluded from this result that residual blasts in the CNS that were not treated with the radiation were able to migrate to the bone marrow and lead to hematologic relapse.

This result clearly demonstrates that, in the context of the systemic chemotherapy employed in this study, cranial radiation is necessary for an optimum outcome. However, since neither the CCG nor the POG nor the MRC routinely utilize cranial radiation, and there is no obvious difference in CNS or hematologic relapse rates among patients studied by these cooperative groups, it is probably the case that cranial radiation can be replaced by intrathecal or systemic chemotherapy. How to accomplish this substitution optimally is not clear, and the issue warrants specific clinical trials. This is an important clinical issue to resolve, since significant morbidity is associated with prophylactic cranial radiation, including both neurocognitive abnormalities and endocrine dysfunction. As AML therapy improves and more children become long-term survivors, the importance of the late effects caused by treatment is magnified.

6.5. What is the Role of Risk-Stratified Therapy Based on Prognostic Factors?

It is clear that the past two decades of AML therapy have been characterized by both an increased intensity of treatment and improved outcomes for children with this disease. It is also clear that increased intensity of therapy carries an increased risk of treatment-related morbidity and mortality. Understanding of cytogenetic abnormalities has progressed as well, and there is retrospective evidence that some patient characteristics portend a good prognosis, whereas others portend a poor one. As AML therapy progresses into the era of targeted therapeutics, it is imperative to identify those patients likely to be cured by conventional therapy and to reserve experimental treatments, at least initially, for those patients who are unlikely to benefit from currently available standard interventions.

Not only do cytogenetic abnormalities have prognostic significance, but there is some suggestion that they might also dictate response to particular therapies. This is most obvious in the case of t(15;17), since involvement of the *RARα* gene is the basis for the use of ATRA for patients with APL. It is also clear that children with trisomy 21 have increased chemotherapy-sensitive disease, particularly to ara-C *(68)*. Beyond these observations, Bloomfield et al. *(36)* published data from adult AML patients demonstrating that the t(8;21) and inv(16) forms of AML are particularly sensitive to ara-C.

There is no consensus regarding the prognostic importance of various patient and leukemic cell characteristics, with different studies revealing different positive and negative prognostic factors. Nevertheless, there are some common findings. Future studies should test directly (in a prospective manner) the prognostic importance of these risk factors and should also test the efficacy of directing therapy based on these prognostic factors, the way that ALL therapy is delivered. Ideally, future stratification of patients will be based on the ability to target putative prognostic factors or pathways. An important issue to consider for the development of these therapies is whether the target in question is in fact relevant to the survival of the leukemic stem cell rather than to its more differentiated offspring.

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CHEMOTHERAPEUTIC STRATEGIES

II

ACUTE MYELOID LEUKEMIA IN ADULTS

I

19 Therapy of Acute Myeloid Leukemia in Adults

BOB LÖWENBERG

CONTENTS

1. INTRODUCTION

Treatment of acute myeloid leukemia (AML) begins with establishing a precise diagnosis. The term AML collectively refers to a mixture of distinct diseases that differ with regard to their pathogenetic evolution, genetic abnormalities, clinical features, response to therapy, and prognosis. Cytogenetic and molecular analyses have been instrumental in identifying disease entities among the mixed bag of AML subtypes, which are currently cataloged as suboptimally defined categories with widely different prognoses. These classifications are mainly based on cytogenetic knowledge. They provide leads in clinical decision making, e.g., with regard to treatment choice. The disclosure of genetic abnormalities may also offer potential targets for treatment intervention.

Today such specific interventions into the molecular intracellular derangements of leukemic cells are only available for exceptional genetically defined entities of AML, such as acute promyelocytic leukemia with the translocation t(15;17). The microarray technology for analyzing differences in gene expression among clinical specimens of leukemia, advances in protein technology, the use of clinically relevant animal models, the development of drug design technology, and the use of appropriate cellular in vitro systems, promise to accelerate our understanding of AML pathogenesis as well as our ability to recognize specific AML disease entities in the near future. With this perspective in mind, what are the current issues in AML therapy?

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2. REMISSION INDUCTION THERAPY

Since the introduction of the anthracyclines (daunorubicin and doxorubicin) and cytarabine, these therapeutic agents have been the cornerstones of remission induction therapy for adult AML *(1)*. With some variations, most centers apply treatment schedules based on these drugs, sometimes supplemented with etoposide. Instead of daunorubicin, some remission induction therapies have incorporated idarubicin *(2–4)*, mitoxantrone *(5–7)*, or amsacrine *(8)*. These combinations induce complete remissions in an average of 70–80% of adults younger than 60-yr. Among patients receiving induction schedules that include idarubicin, fewer individuals relapse, and overall survival also appears to be slightly better than with other regimens *(2–4,9)*, although it is questionable whether equitoxic dosages of idarubicin and daunorubicin were compared in these studies.

Table 1
Randomized Studies of Autologous Bone Marrow Transplantation (ABMT) in Acute Myeloid Leukemia in First Remission

Study	*Comparison*	*Median age (yr)*	*No. (%) of patients randomized*	*% Patients assigned to ABMT who were actually transplanted*	*DFS*	*OS*
EORTC-GIMEMA 1995 *(14)*	ABMT vs CT	33	254 (63)	74	ABMT > CT	ABMT = CT
GOELAM 1997 *(19)*	ABMT vs CT	36	164 (61)	87	ABMT = CT	ABMT = CT
POG 1996 *(18)*	ABMT vs CT	Children	232 (50)	62	ABMT = CT	ABMT = CT
MRC 1998 *(15)*	ABMT vs none	32	381 (34)	66	ABMT > CT	ABMT = CT
HOVON-SAKK 2000 *(43)*	ABMT vs none	44	128 (46)	68	ABMT = CT	ABMT = CT
Intergroup 1999 *(16)*	ABMT vs CT	NR	346 (69)	54	ABMT = CT	ABMT = CT

Abbreviations: DFS, disease-free survival: OS, overall survival; CT, chemotherapy; none, no further chemotherapy; NR, not reported; EORTC-GIMEMA, European Organization for Research and Treatment of Cancer-Gruppon Italiano Malattie Ematoligiche Maligne dell'Adult; GOELAM, Groupe Ouest Est Leucemies Aigues Myeloblastiques; POG, Pediatric Oncology Group; MRC, Medical Research Council; HOVON-SAKK, Hemato-Oncology Research Group.

Circumstantial evidence would indicate that dose intensification of induction therapy may improve the quality of remission in adults with AML. Thus, for instance, the duration of remission might be increased by including high-dose cytarabine in the initial cycles of induction therapy *(10,11)*. Alternative approaches to remission induction treatment (e.g., drug resistance modulation, anti-AML targeted therapy) are also being explored. When combined with conventional chemotherapy, such strategies may lead to more frequent remission induction or to remissions of longer duration. Today, most complete responders relapse within 2 yr following diagnosis, so that treatment development has concentrated on postremission therapy.

3. POSTREMISSION THERAPY: INTENSIVE CHEMOTHERAPY WITH OR WITHOUT AUTOLOGOUS STEM CELL TRANSPLANTATION

During the last 20 yr there has been a shift from low-dose maintenance chemotherapy administered for prolonged times (1–2 yr) toward intensified cycles of chemotherapy delivered within a concentrated period (4–6 mo). These dose-escalated and time-condensed cycles are given once a complete remission is induced and serve the objective of eradicating minimal residual leukemia. Most commonly, these regimens are based on high-dose cytarabine without autologous stem cell reinfusion *(12,13)* or on high-dose cytotoxic therapy followed by hematopoietic stem cell transplantation *(14–16)*.

Survival rates in large phase III studies of high-dose chemotherapy for AML patients 60 yr of age or younger have been estimated at 40–55% at 4 yr. These results would indicate a dose-response relationship for chemotherapy in patients with AML *(10–16)* In one study, the results suggested that patients with t(8;21)-positive AML may benefit from escalated doses of cytarabine *(17)*. High-dose cytotoxic therapy followed by autologous stem cell transplantation (auto-SCT) *(14,16,18,19)* produces a similar outcome. In these trials, auto-SCT was compared with either no further postremission treatment or "conventional-type" postremission chemotherapy (Table 1). In certain studies *(14,15)* but not others *(16,18,19)*, disease-free survival was improved after auto-SCT owing to some reduction in the probability of relapse. However, in none of these studies was there a significant advantage in overall survival favoring auto-SCT (40–55% at 4 yr), most likely because a proportion of patients relapsing after chemotherapy can be rescued by an autograft in second remission.

Hematopoietic recovery after auto-SCT has generally been slow, leading to considerable transfusion requirements postgrafting and to some hemorrhagic and infectious deaths *(20)*. As a result, the procedure-related mortality following auto-SCT has been somewhat greater than after chemotherapy, offsetting the advantage of the reduced relapse frequency associated with autologous transplantation. Furthermore, only a limited fraction of complete responders proceed to transplantation. Premature withdrawal from autografting is the consequence of the harvest of an insufficient number of hematopoietic cells for grafting, intercurrent infections, or early relapse of leukemia. The introduction of peripheral blood progenitor cell autografts may circumvent these problems and contribute to a broader applicability of autografting in AML with less toxicity *(21)*.

An important question that remains to be resolved is whether certain subgroups of patients with AML benefit more from auto-SCT than do others. There is evidence suggesting that patients with intermediate-risk AML (according to cytogenetics) derive more benefit from auto-SCT than from intensive chemotherapy alone *(15)*, but this has not been confirmed in other studies *(16,19)*. Definite conclusions regarding the potential benefit of autologous stem cell transplantation in distinct prognostic subsets of AML will require additional studies enrolling larger numbers of patients.

4. POSTREMISSION THERAPY: ALLOGENEIC STEM CELL TRANSPLANTATION

Allogeneic stem cell transplantation (allo-SCT) following myeloablative cytotoxic therapy currently offers the most powerful antileukemic treatment modality for adults with AML in remission *(22–24)*. When an HLA-matched allogeneic sibling donor is available, the option of allo-SCT is usually the first choice. In patients with AML in complete remission (CR) receiving an allogeneic stem cell graft, the probability of relapse is significantly reduced *(23–27)*. The risk of relapse in patients with AML in first CR following transplantation of an HLA-matched sibling allograft may vary from 10 to 25%. Accumulating evidence suggests that disease-free survival and overall survival are also slightly better following allo-SCT *(14,23,24)*, although this observation has not always been consistent *(16)*.

The advantage of a reduced probability of relapse of AML after allo-SCT is partially lost owing to enhanced procedure-related mortality (caused by acute and chronic graft-versus-host disease and posttransplant immunodeficiency complicated by interstitital pneumonia and serious opportunistic infections). As the application of an allograft is practically dependent on the availability of a fully matched family donor and specific age eligibility limitations, comparisons of outcome following allografting and autografting or chemotherapy have not been based on true randomizations. More recently, investigators have compared outcome between patients with an HLA-matched donor (regardless of whether or not the transplant was done) and those without an available donor in an effort to mimic an intent-to-treat evaluation. The results suggest reduced relapse rates for allotransplanted patients with AML in first complete remission (unpublished data).

Considering the clinical heterogeneity of AML, an important issue has been whether certain subsets of patients benefit more from an allograft than do others. For instance, in patients with good-risk AML (based on cytogenetics) with an *a priori* risk of relapse of ≤25%, one might prefer to avoid an allograft in first complete remission and avoid the associated enhanced death rate. As a matter of fact, patients with good-risk AML have a greater chance of being rescued in case of relapse. This argues for a delayed allotransplant strategy only in selected cases of recurrence in good-risk AML. In contrast, intermediate-risk patients with greater relapse probabilities (40–50%), as well as poor-risk patients with comparatively high relapse rates (80%), might be "preferred" candidates for an allograft as early as possible. In these individuals, the value of the greater antileukemic efficacy of allografting would more likely outweigh the risk of greater transplant-related toxicity and mortality *(8,26)*. However, data on the relative value of allo-SCT in cytogenetically defined prognostic subgroups of AML are still scarce.

HLA-matched unrelated donor transplants are increasingly employed when a genotypically HLA-matched donor is not available. They are associated with enhanced risks of transplant-related complications and death. Although such transplants are mainly applied to restricted categories of high-risk cases (poor-risk AML in CR1, or AML in CR2 or CR3 or in early relapse) their value remains to be critically assessed in large series of patients.

5. TREATMENT OF OLDER PATIENTS

Most patients with AML are 60 yr of age or older. Although treatment results have improved steadily in younger adults over the last 20 yr owing to the application of more intensive cytotoxic treatment, the use of stem cell grafts, and improvements in supportive care, there have been no significant changes in outcome among individuals of 60+ yr of age. When treated with chemotherapy alone, this subgroup has an estimated 2-yr survival of approximately 20% *(28)*, compared with 10% or less at 4–5 yr *(7,29,30)*.

Epidemiologic data indicate that the outcome of older subjects is worse than these estimates would indicate, as many older patients are not referred to hospitals for treatment and are not registered or evaluated for outcome. Why is outcome so unsatisfactory in the aged? The reasons probably relate to the increased frequency of unfavorable cytogenetics among older patients with AML, and a greater frequency of antecedent myelodysplasia, and a greater frequency of drug resistance phenotypes *(31,32)*. Finally, because of the reduced quality of their general health, older patients cannot tolerate intensive chemotherapy as well as younger individuals do.

Bone marrow transplants are infrequently done in patients 60 yr of age or older *(33)*. A randomized study of postremission therapy comparing cytarabine schedules at three dose levels (100 mg/m^2, 400 mg/m^2, and 3 g/m^2) showed a dose-effect relationship as regards (disease-free) survival in adults younger than 60 yr, but not in older subjects, that is, the schedule of cytarabine at 3 g/m2 resulted in a reduced relapse probability and superior survival in patients 60 yr old or younger *(12)*, whereas an advantage of cytarabine dose escalation was not apparent in older individuals. Thus, high-dose chemotherapy does not appear to be beneficial in the elderly with AML. Since such patients do not tolerate intensive chemotherapy, there has been an increasing interest in the development of allotransplantation following chemotherapy with non-myeloablative preparative regimens. The goal of these approaches is to establish allogeneic chimerism following immunosuppressive therapy and then exploit the graft-versus-leukemia effects of the allografts, so that donor chimerism can be used as a platform for subsequent infusions of donor lymphocytes. Early clinical trials afford proof of principle of this approach, but for the time being they are based on small patient numbers and have limited follow-up. In older patients with various hematologic malignancies and (mixed) donor chimerism can be established, but more mature data will be needed for a critical assessment of the clinical value of this strategy *(34)*.

6. PROGNOSTIC FACTORS

AML arises from a malignantly transformed hematopoietic stem or progenitor cell. The stage of cell development (more or less primitive, multipotent, or committed) at which transformation occurs and the residual capabilities of maturation of these cells determine the phenotypic diversity of the disease *(35–37)*. These variations are reflected in the different cytomorphologic features of the leukemic blasts, the dysplasia of the other hematopoietic cell lineages, and the characteristic sites of leukemia presentation (e.g., skin, gingiva, and so forth). The

molecular abnormalities leading to full leukemic transformation vary greatly. Differences in molecular pathogenesis may also dictate the variation of clinical presentation as well as the response to therapy.

Understanding of the prognostic determinants of response to therapy in patients with AML is still evolving. Cytogenetic analysis has become part of the essential standard workup of patients with AML and currently furnishes distinctive insights into the nature of the disease, providing useful clues to the prognosis of individual patients. However, more precise distinctions are needed to provide better quantitative prognostic estimations and to establish elementary guidelines for treatment choice.

6.1. Age

Age has a strong impact on the outcome of AML. This prognostic relationship is apparent as a progressive decrease in treatment response and survival as a function of age from infancy to 85 yr of age (Collaborative AML Intergroup Oxford, personal communication). For practical reasons of protocol design, a cutoff limit is usually set at 60 yr to distinguish older patients. This subgroup has an overall probability of response to induction therapy of 40–50%, and <10% of these responders survive beyond 5 yr following diagnosis *(7,30)*.

6.2. Cytogenetics and Molecular genetics

Cytogenetic abnormalities are seen in approx 60% of cases of AML and are highly predictive of response to therapy as well as the probability of relapse *(38–41)*. Translocations t(8;21), inv16 or t(16;16), and t(15;17) generally carry a relatively favorable prognosis and are more commonly seen among younger patients with AML. The fusion genes of each of these translocations have been identified and can be detected with molecular probes in reverse transcriptase polymerase chain reaction (RT-PCR). Among patients with these chromosomal abnormalities or the corresponding molecular genetic abnormalities (*AML1-ETO*, *CBFβ-MYH11*, *PML-RAR-α*), the response to induction therapy is ≥80%, and for those entering remission the probability of relapse is approximately 30%, resulting in 5-yr survival rates of 60–70%. The latter molecular or cytogenetic subsets of AML correlate with certain cytomorphologic categories. For instance, t(8;21) correlates mainly with the FAB subtype M2, whereas inv16 correlates with FAB M4 with eosinophils. However, the cytogenetics and molecular genetics of a particular case, rather than the FAB subtype, possess the overriding prognostic significance.

In contrast, patients with monosomies of chromosomes 7 (–7) or 5 (–7), 11q23 (*MLL*-gene) abnormalities *(42)*, abnormalities of the long arm of chromosome 3 (abn3q), t(6;9), and complex cytogenetic abnormalities (four or more different cytogenetic aberrations) generally have a distincly poor prognosis. Among these individuals (when 60 yr of age or younger), the average complete response to induction treatment is 60%,with most patients (approx 80%) relapsing within 2 yr so that the survival rate at 5 yr is approx 15% *(8,26,27)*. These cytogenetic and molecular indicators have led to the definition of prognostic groups: favorable risk (favorable molecular genetics or cytogenetics), unfavorable risk (poor-risk cytogenetics), and intermediate risk (all others). Usually, these risk classifications are refined by considering selected additional

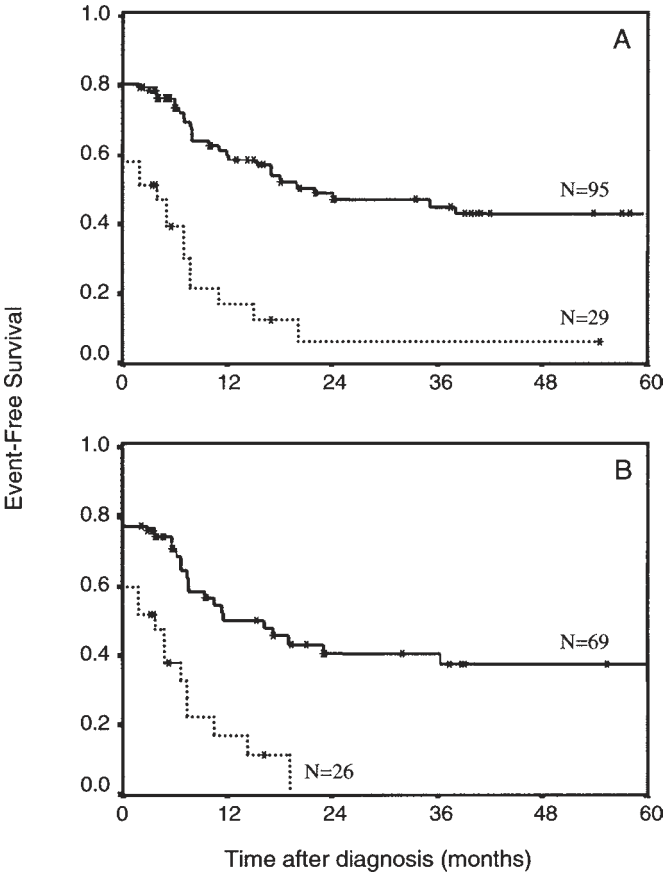

Fig. 1. Effect of *Flt3* gene mutations (*FLT3*/ITD) on event-free survival of adult AML patients. Solid lines indicate *Flt3*/ITD (internal tandem duplication) negative samples. Dotted lines refer to *Flt3*/ITD-positive population. **(A)** Actuarial event-free survival of the total AML population. **(B)** Event-free survival of the AML population with intermediate-risk cytogenetics. Censored cases are marked with an asterisk.

prognostic determinants that enhance the value of these cytogenetic or molecular distinctions. The risk-scoring systems based on multiple prognostic factors may permit more precise approximations of prognosis in individual cases. For the sake of practicality, most collaborative groups have restricted the use of these additional risk factors and have only taken into account a limited number of covariables with considerable prognostic impact. As an example, the Medical Research Council (MRC) group has considered the rapid vs late attainment of complete response (i.e., after cycle 1 vs cycle 2) in their risk score in order to enhance the separation between intermediate-risk and poor-risk patients *(27)*. The Dutch–Belgian Hemato-Oncology Cooperative Group (HOVON) and Swiss SAKK groups have required a white blood cell count of $<20 \times 10^9$/L in addition to favorable cytogenetics for the good-risk category (risk of relapse, 24% at 5 yr) (Fig. 1). The current prognostic scoring system of the HOVON/SAKK Leukemia Groups is presented in Table 2.

6.3. Proliferative Abilities of AML Cells In Vitro and Growth-Factor Receptor Mutations

It has been shown that autonomous proliferation of AML cells in short-term culture predicts a relatively high probability

Table 2
Prognostic Scoring System for Patients with AML in First Complete Remission (According to the HOVON Cooperative Group)[a]

Risk Group	*No. of cases (%)*	*5-Yr probabilities*		
		Relapse (%)	*Death in first CR (%)*	*Overall survival (%)*
Good risk				
AML with t(8;21)(q22;q22) or *AML1/ETO* fusion transcript[b]	86 (10)	28	11	74
With WBC ≤ 20×10^9/L at diagnosis				
Without additional unfavorable cytogenetic abnormalities[c]				
or				
AML with inv/del(16)(p13q22) or *CBFβ/MYH11* fusion transcript[b] without additional unfavorable cytogenetic abnormalities				
Intermediate risk				
Any AML case not assigned to a good-risk or poor-risk group	474 (57)	50	10	44
Poor risk				
AML with unfavorable cytogenetic abnormalities (except those with simultaneous favorable cytogenetic abnormalities)	207 (25)	70	12	22
or				
AML without favorable and without unfavorable cytogenetic abnormalities; includes patients with no cytogenetic abnormalities and those without cytogenetics done, but with a late CR	70 (8)	80	9	24

Abbreviations: early CR, complete remission attained after induction cycle 1; late CR, complete remission attained after induction cycle 2; WBC, white blood cells.

[a]Prognostic score is based on landmark analysis of data from HOVON-SAKK trials AML-4 and AML-29 and does not consider AML FAB-type M3 with t(15;17) or the *PML-RARa* fusion gene.

[b]Favorable cytogenetics include both t(8;21)(q22;q22)/*AML1-ETO* and inv/del(16)(p13q22)/*CBFβ-MYH11*.

[c]Unfavorable cytogenetics include complex cytogenetic abnormalities (more than three distinct clonal abnormalities); 7,-5 (monosomies of chromosomes 5 or 7); del 5q or del 7q (deletions of the long arm of chromosomes 5 or 7); abnormalities of the long arm of chromosome 3 (abn 3q); t(6;9)(q23;q24)/*DEK-CAN* fusion gene; and abnormalities of the long arm of chromosome 11 (abn 11q23).

of relapse and poor survival *(44)*. Patients with AML and high spontaneous blast cell proliferative activity also more frequently express CD34 positivity and a multidrug resistance phenotype *(45)*. More recently, mutations in the genes encoding receptors for hematopoietic growth factors have been detected, in particular, in *Flt3* and *kit (46–51)*.

Fms-like tyrosine kinase 3 (Flt3, receptor for FL or flt ligand), kit (receptor for stem cell factor or kit-ligand), and fms [receptor for macrophage colony-stimulating factor (M-CSF)] are class III receptor tyrosine kinases that play a role in hematopoiesis and share similar structural domains. Both *kit (52,53)* and *fms (54)* are expressed in a significant proportion of AML cases and may carry mutations. *kit* mutations are mainly seen in patients with good-risk cytogenetics *(55,56)*. Internal tandem duplications of *Flt3* have been reported in the part of the *Flt3* gene coding for the juxtamembrane (JM) segment of the receptor in 15–20% of AML cases *(50,51)*. The elongation of the JM domain causes ligand-independent dimerization, resulting in constitutive activation and a reduced responsiveness to stromal support in long-term culture *(57)*. *Flt3* mutations have been shown to confer a grave prognosis in both young and older adults, independent of other prognostic factors. Of particular note is the large subset of cytogenetically defined intermediate-risk patients, who can be split into good-risk and poor-risk subgroups according to the absence or presence of *Flt3* gene mutations *(50)* (Fig. 1).

7. DRUG RESISTANCE

By definition, patients who relapse or fail to respond to current chemotherapy programs show clinical drug resistance. There is much interest in the mechanisms of drug resistance and the extent to which they play a role in AML treatment responsiveness. Resistance of AML to the cytotoxic effects of chemotherapy has remained the major stumbling block to cure. Thus, one would assume that insights into the molecular pathways leading to drug resistance might provide particularly powerful prognostic markers of response to therapy. Multidrug resistance type I (MDRI) or classical drug resistance is associated with the enhanced expression of P-glycoprotein (Pgp), an ATP-binding drug transporter in the plasma membrane of leukemic cells. This transporter may bind a variety of substrates (including anthracyclines and epipodophylotoxins) and acts as an efflux pump for these drugs.

High levels of MDR1 have been associated with reduced intracellular concentrations of chemotherapy within leukemic cells. The frequency of MDR1-positive AML increases markedly with age *(32,58)*. In several studies MDR1 positivity has been shown to have negative prognostic value for response to induction chemotherapy *(32,45,58–61)*. This relationship was seen in studies in which MDR1 was assessed by Pgp staining or by mRNA measurements, but not in other studies *(62)*. It should be noted that a lack of agreement among some studies may relate to technical variations (e.g., use of different end

points for positivity), differences in cell sample preparation (e.g., fresh vs cryopreserved) and data analysis, and use of different immunologic reagents *(63)*. In some studies, functional assays of drug export (e.g., rhodamine export inhibitable by an MDR blocker) have disclosed prognostic significance for this variable *(58,64,65)*. Of particular interest are the results of a study in which simultaneous functional measurements of MDR-associated protein 1 (MRP1) and Pgp activity showed a strong correlation with response to treatment, primarily among the subgroup with adverse cytogenetics *(65)*.

Notably, in most of these studies, the prognostic significance of MDR1 was evaluated in the context of daunorubicin chemotherapy. In a study in patients treated with induction chemotherapy including idarubicin, there was no correlation between MDR1 expression and response *(66)*. It is not unlikely that multiple resistance mechanisms cooperate in the same patients and determine clinical resistance through synergistic interactions. This hypothesis is supported by the recent observation of two distinct resistance phenotypes, which in combination added to the prognostic impact of the individual marker *(65)*. Finally, it appears of particular interest to relate resistance phenotyping to specific subpopulations of AML cells, i.e., AML progenitor cells rather than the overall AML cell population. In one study the assessment of MDR1 expression on the subpopulation of CD34-positive cells by dual surface marker analysis significantly enhanced the predictive value of this marker *(45)*.

Other genes involved in the cellular redistribution of chemotherapeutic agents are MRP, a transporter of the glutathione complex, and the lung resistance protein (LRP). Two studies indicated that LRP may predict for response as well as leukemia-free survival *(65,67,68)*, but others did not reveal a correlation between expression of LRP and response *(58,69)*. One of the pathways of apoptosis is critically regulated by members of the Bcl-2 superfamily. Bcl-2 may be overexpressed in AML with no clear association with FAB cytologic classification or with cytogenetics. High Bcl-2 expression has been shown to predict for poor response to chemotherapy in some but not all studies *(70–74)*.

Although the molecular pathways leading to the development of drug resistance in AML remain largely unknown, drugs able to reverse resistance are currently being developed. However, early studies with inhibitors of Pgp function (e.g., cyclosporin A and its analogs) have yet to fulfill their therapeutic promise *(75,76)*.

8. HEMATOPOIETIC GROWTH FACTORS

The use of hematopoietic growth factors to accelerate hematopoietic recovery and prevent morbidity has attracted wide attention. Granulocyte (G)-CSF and granulocyte-macrophage (GM)-CSF can stimulate the production of granulocytes (both G-CSF and GM-CSF) and monocytes (GM-CSF), promote their mobilization from the marrow to the blood circulation, and activate granulocyte and monocyte function. Thrombopoietin and several other new cytokines have more recently become available for clinical investigation in patients with AML. A substantial number of randomized studies have been completed evaluating the application of G-CSF or GM-CSF as adjuncts to induction or consolidation cycles of chemotherapy *(77)*. The duration of neutropenia was consistently shorter with use of either cytokine in these studies. This benefit translated into fewer days of antibiotic *(78–80)* or antifungal therapy *(81)* or fewer days in the hospital *(81)* in some but not most studies. None of the studies showed a reduction in the number of documented infections. In one comparative study, survival appeared to be improved in the GM-CSF treatment group *(82)*.

The findings do not warrant routine use of G-CSF and GM-CSF in the clinical management of AML patients. However, the therapeutic use of these cytokines might be justified in patients with serious infections unresponsive to antimicrobial treatment. A future role for myeloid CSFs is suggested by studies in which peripheral blood progenitor cell autografts (to replace marrow transplants) showed accelerated hematopoietic regeneration following mobilization with CSFs and cytapheresis *(21)*.

9. MINIMAL RESIDUAL DISEASE

The genetic abnormalities seen in a significant proportion of cases of AML provide unique markers of minimal residual disease, which in some instances can be detected with molecular methods. Such markers have been used to monitor the disappearance of leukemic cells during treatment and during remission, or to assess their reemergence following remission. Conventional cytogenetic analysis, fluorescence *in situ* hybridization, and Southern blot analysis provide relatively insensitive measurements (1 leukemic cell/10^2 cells). RT-PCR measures transcripts of fusion genes at much greater sensitivity (10^{-6}–10^{-3}) and, more recently, real-time PCR has been introduced for quantitative measurements of fusion gene transcripts. As yet, these techniques have been applied mainly to the most common fusion genes: *AML1-ETO* [t(8;21)], *CBFβ-MYH11*, [inv16, t(16;16)], *BCR-ABL* [t(9;22)], and a few others.

In AML with t(8;21), *AML1-ETO* fusion transcripts have been detected in patients in long-term remission, indicating that their persistence does not necessarily predict for relapse *(83–86)*. A proportion of the long-term survivors did become negative for such transcripts *(87)*. More recently, quantitative PCR techniques have permitted individuals at high risk of relapse to be distinguished from those in stable remission *(88,89)*. These studies are all based on small series of cases, emphasizing the need for more information regarding the predictive value of quantitative PCR measurements. Studies of real-time PCR are in progress.

In AML with the inv(16) or t(16;16), *CBFβ-MYH11* transcripts have been followed. There are at least eight variant transcripts. Most studies conducted so far have only assessed the common type A transcript (present in >80% of patients with this abnormality) in limited numbers of patients and therefore cannot provide definite conclusions regarding the clinical value of this marker *(89–94)*. Both PCR-positive and PCR-negative cases have been reported among long-term survivors. Studies with quantitative PCR have as yet provided preliminary data only *(95,96)*.

Another significant proportion of patients with AML present with *MLL* gene rearrangements (abn 11q23) *(42)*. One of many *MLL* abnormalities is the *MLL-AF9* fusion gene in patients with the t(9;11) translocation, but the value of molecular methods for the detection of this genetic abnormality remains uncertain *(97,98)*.

10. TARGETED LEUKEMIC CELL KILLING

An appealing concept of AML treatment is to direct therapeutic agents specifically to the leukemic cell population while sparing normal hematopoietic progenitors and normal tissue. Antibodies and growth factors conjugated to a cytotoxic agent can be used to deliver drugs to the site of the malignant cell. For instance, the GM-CSF/cholera toxin or the GM-CSF/*Pseudomonas* toxin have shown significant activity against leukemic progenitor cells in colony assays as well as in vivo repopulation assays *(99,100)*. The CD33 antigen is a 67-kDa glycoprotein that functions as a sialic acid-dependent adhesion protein. It is expressed on most AML cells, including leukemic clonogenic precursors but is not expressed on normal pluripotent hematopoietic stem cells. The CD33 antigen is absent from nonhematopoietic tissue. Because of these properties of selective binding and the fact that upon binding of specific antibody the CD33 antigen is internalized, anti-CD33 antibodies are suitable for delivering cytotoxic agents to leukemic cells *(101,102)*. Several other interesting immunoconjugates (e.g., anti-CD45 antibodies and G-CSF/toxin conjugates) are under development. Early clinical studies with the humanized antimyeloid CD33 antibody conjugated with the chemotherapeutic antitumor antibiotic calicheamycin (gemtuzamab ozogamicin) show that approximately 30% of cases with AML in relapse may enter complete remission after one or two injections of this agent *(101)*. So far, experience with this drug as a single agent is restricted to patients with advanced disease.

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20 Treatment of Adult Acute Myelogenous Leukemia

RICARDO T. SPIELBERGER AND STEPHEN J. FORMAN

CONTENTS

1. INTRODUCTION

Historically, the classification and treatment of acute myelogenous leukemia (AML) has been based on morphologic and clinical observations. The identification of the molecular events involved in the pathogenesis of human tumors, including the acute leukemias, has refined their classification and understanding. In AML, a large number of leukemia-specific cytogenetic abnormalities have been identified and the involved genes cloned. These studies have helped elucidate the molecular pathways that may be involved in cellular transformation and have provided methods for the monitoring of patients after chemotherapy as well as evaluating treatment responses based on various clinical, phenotypic, and genetic risk factors. Table 1 lists the consistent cytogenetic abnormalities found in AML and their unique gene products *(1)*. Although the leukemic cells in many patients do not have detectable structural chromosomal changes at diagnosis, some may harbor molecular changes not apparent on routine karyotyping such as those involving the *MLL* gene *(2)*. Taken together, these observations have led to the concept that AML is, in fact, a heterogeneous disease with its variants best defined by their molecular defects. Whereas in previous clinical trials of standard chemotherapy, or of allogeneic and autologous transplantation, patients were often treated as a homogeneous group, recent studies have refined the way patients are allocated to various treatments as well as the analysis of the results.

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2. DIAGNOSTIC CONSIDERATIONS

As previously noted, the primary diagnosis of AML has relied on morphologic criteria to identify leukemic myeloblasts in the peripheral blood and/or bone marrow. Currently, the presence of >30% leukemic blasts in the bone marrow aspirate is required for the definitive diagnosis of AML; however, several critical distinctions should be made to distinguish the disease from acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), or AML arising in the setting of MDS. Although the therapeutic approach to patients in whom leukemia arises out of myelodysplasia often has a background of dysplastic changes seen in the erythroid and myeloid series, this by itself is not an infallible basis for the distinction. This morphologic finding, however, combined with a history of an antecedent hematologic abnormality, such as a chronic anemia, leukopenia, or thrombocytopenia, provides evidence of antecedent dysfunction of the marrow and indicates a probable different pathogenesis for the disease. In general, AML can be distinguished from ALL by immunohistochemical demonstration of a definitive commitment to myeloid differentiation. The distinction of AML from MDS has been recently modified by a recent change in the World Health Organization (WHO) classification, suggesting that patients with >20% blasts at diagnosis be considered as having AML.

Cytogenetic analysis of the diagnostic specimen is the most important test not only in the diagnosis of AML, but also in establishing a prognosis for the patient and in determining the treatment options. As described below, the risk category of leukemic cytogenetic abnormalities will determine the likelihood of success for both remission and postremission therapy

Table 1
Frequent Recurrent AML-Associated Cytogenetic Abnormalities in Adults

Cytogenetic abnormality	*Frequency in adults (%)*	*Critical fusion genes*	*Associated FAB morphologic subtypes*
t(8;21)(q22;q22)	5–12 (<45 yr), rare (>45 yr)	*AML1-ETO*	>80% M2; also M1, M4
inv(16)(p13q22)	10 (<45 yr), rare (>45 yr)	*CBFβ-MYH11*	>95% M4Eo; also M2, M4
t(16;16)(p13;q22)			
t(15;17)(q21;q11)	15 (<45 yr), rare (>45 yr)	*PML-RARα*	M3 M3v
Variants			
t(11;17)(q23;q11)		*PLZF-RARα*	M3, M3v-like
t(5;17)(q32;q11)		*NPM-RARα*	M3, M3v-like, APL
del(11)(q23)			
11q23 translocations	5–7	*MLL*	Predominantly M4, M5a; other FAB subtypes (M1, M2, M5b) or MDS more rarely associated
t(4;11)(q21;q23)		*MLL-AF4*	
t(9;11)(p22;q23)		*MLL-AF9*	
t(11;19)(q23;p13.1)		*MLL-AFX*	
t(11;19)(q23;p13.3)		*MLL-ELL*, *MLL-ENL*	
t(8;16)(p11;q13)	<1	*MOZ-CBP*	M5b/M4
t(11;16)		*MLL-CBP*	
t(11;22)		*MLL/p300*	
t(6;9)(p23;q34)	Rare	*DEK-CAN*	M2, M4
inv(3)(q21q26), t(3;3)(q21;q26)	3–5	*Ribophorin-EV11*	MDS;M0;M1; all FAB subtypes: M7
t(1;22)(p13;q13)	Rare		M7
Poor prognosis and complex abnormalities	10–15 (<45 yr), 30–40 (>45 yr)	Unknown	All FAB types; more frequently M1, M1, M2
5/5q–, –7/7q–, 17p abn or i(17q),del(20q), dmins, hsrs, +13, complex			

Abbreviations: FAB, French-American-British classification system; dmins, double minute chromosomes; hsrs, homogenously staining regions; del, deletion; abn, abnormality; inv. inversion; t, translocation.

and thus long-term prognosis. In addition to cytogenetics, other variables have consistently been found to predict for poor disease-free survival including older age, high white blood cell count at diagnosis, dysmyelopoiesis, or more than one cycle of induction chemotherapy to achieve a remission. Some investigators have noted that the expression of CD34 on blast cells or the expression of the multidrug resistance (*MDR*) gene at the time of diagnosis also suggests a poor prognosis. Table 2 delineates the poor prognostic features of patients with AML treated with chemotherapy alone *(3–13)*.

The molecular cloning and characterization of the chromosome abnormalities seen most frequently in AML include the 8;21, and 15;17 translocations and inversion 16. Preliminary studies indicate that molecular testing is an important technique to monitor the long-term outcome of patients undergoing therapy *(14)*. This has been most importantly demonstrated in patients with acute promyelocytic leukemia (*see* Chapter 1), in whom the achievement of a polymerase chain reaction (PCR)-negative state for the presence of the 15;17 translocation translates into longer disease-free survival, whereas its persistence predicts relapse. Translocation of the 8;21 gene is one of the most frequently seen abnormalities in AML, occurring in up to 15% of patients. Patients with this cytogenetic abnormality have a good response to chemotherapy, with higher remission rates and long-term survival after a variety of therapeutic approaches *(14,15)*. Although studies reporting the molecular monitoring of AML patients with the 8;21 translocation are limited, they do suggest that nonquantitative reverse transcriptase (RT-PCR) methods do not have a predictive value in detecting minimal residual disease *(16)*. Patients who have the inversion 16 (p13, q22), which is also associated with a good prognosis and high remission rate, can also be followed with RT-PCR. The predictive value of this assay is uncertain at present, but in general it appears that long-term, disease-free survival is associated with RT-PCR negativity *(17,18)*.

The major difficulty in minimal residual disease detection in patients with AML derives from the observation that approximately half the patients have no detectable cytogenetic abnormality ("normal karyotype"). Recent progress in the characterization of unknown chromosome abnormalities is increasing the number of cases with a detectable clonal

marker *(19)*. These studies also indicate that some of the cytogenetically normal cases will be found to have abnormalities (translocations and partial tandem duplications) of the *MLL* gene located at 11q23. Patients with these abnormalities have a poor prognosis because of the short duration of remission, helping to account for the inferior prognosis of the intermediate-risk group of patients with AML with normal karyotypes. From a diagnostic perspective, the use of DNA microarray techniques has been demonstrated to segregate those patients with myeloid or lymphoid differentiation, and it will probably become part of the diagnostic panel used to categorize the acute leukemias, particularly those with unclear surface immunophenotypes *(20)*.

3. THERAPEUTIC APPROACHES

3.1. Induction Therapy

The ideal induction chemotherapy regimen for adult AML provides rapid attainment of a complete remission with one course of induction therapy in a high percentage of patients. This objective should be accomplished with low toxicity to the nonhematopoietic system, allowing patients to proceed expeditiously to postremission therapy, without which nearly all patients will succumb to recurrent leukemia. Ideally, the regimen should be able to overcome identifiable poor-risk factors, offer an improved complete remission rate, and improve remission duration when followed by intensive postremission therapy. As noted above, the most significant predictor for achieving a remission is the presence or absence of cytogenetic abnormalities at diagnosis. The impact of such abnormalities on the ability both to achieve and to sustain remission was first recognized in the mid-1980s based on 290 patients with *de novo* AML treated with standard dose cytarabine and daunorubicin.

The Fourth International Working Group on Chromosomes and Leukemia discerned that >80% of patients with (8;21) or inversion 16 consistently achieved a complete remission (CR), whereas patients with either a loss or gain of whole chromosomes or –5q or –7q had a <50% chance of remission. Patients without detectable abnormalities (i.e., normal karyotypes), 11q abnormalities, or translocations of chromosomes 15 and 17 had intermediate remission rates of 60–70%. Recent data from a study conducted by the Southwest Oncology Group and the Eastern Cooperative Oncology Group indicated that karyotypic analysis predicted the outcome of remission induction therapy consisting of 7 d of cytarabine and 3 d of daunorubicin. Of 584 patients, 412 (71%) achieved a remission *(21)*. The CR rate among the three groups varied significantly according to known cytogenetic risk status, ranging from 84% (98 of 117) for favorable to 76% (205 of 270) for intermediate and 55% (96 of 173) for unfavorable. Thus, the heterogeneity of response was largely owing to the lower CR rate in the unfavorable group compared with the other two groups combined. The difference between the intermediate- and favorable-risk groups was not significant. Multiple logistic regression analyses of the prognostic effects of the available pretreatment variables have suggested that cytogenetic risk status and performance status at the time of presentation are the most significant prognostic factors for response.

Table 2
Poor-Risk Factors in Patients with AML Treated with Chemotherapy Alone

1.	Older age
2.	Poor-risk cytogenetics
3.	High WBC count at diagnosis
4.	CD34 expression
5.	*MDR1* expression
6.	FAB M0, M1, M5, M6, M7
7.	Secondary AML (therapy-related or developing after MDS)
8.	Dysplastic morphology at diagnosis
9.	Extramedullary disease at diagnosis
10.	More than one cycle of induction chemotherapy required to achieve complete remission

Abbreviations: FAB, French-American British classificaiton; MDS, myelodysplastic syndrome; WBC, white blood cell.

The German AML Cooperative Group compared two courses of cytarabine, daunorubicin, and 6-thioguanine with one course of this therapy followed by high-dose cytarabine (HDAC) and mitoxantrone for induction therapy *(22)*. The CR rate was similar for both groups (65% vs 71%); however, patients with unfavorable karyotypes who received HDAC had an improved CR rate ($p = 0.011$). This was the first randomized study to demonstrate an improved CR rate for a subgroup of AML patients treated with HDAC induction. Updated results of the Australian Leukemia Study Group, now with a median duration of follow-up of 5.7 yr, reported equivalent CR rates in their comparison of HDAC vs standard cytarabine, daunorubicin, and etoposide; however, the estimated median duration of remission with HDAC was 46 mo and 12 mo with standard cytarabine. Relapse-free survival was 48% vs 25% in the two groups. The median survival after CR was 38 mo with HDAC vs 22 mo with standard cytarabine *(23)*. The Southwest Oncology Group trial suggested that patients receiving high-dose cytarabine induction had improved disease-free survival, whereas the Cancer and Leukemia Group B (CALGB) comparison of high-dose cytarabine vs intermediate- or standard-dose cytarabine indicated that HDAC consolidation also improves disease-free survival *(24)*. Thus, at the present time, it appears that treatment with HDAC, during either induction or consolidation, has a favorable impact on disease-free survival. At the City of Hope, we treated 122 patients who had *de novo* non-M3 AML with a regimen of HDAC (3 g/m^2 given over 3 h every 12 h for a total of eight doses) followed by daunorubicin (60 mg/m^2 daily for 2 d) *(25)*. A CR was induced in 80% of the patients, whereas 16% had refractory disease and four died of sepsis during hypoplasia. The CR rates for favorable, intermediate, and unfavorable cytogenetic groups were 87, 79 and 62%, respectively. High white blood cell count at diagnosis, older age, unfavorable French–American–British (FAB) subtype, and high lactate dehydrogenase (LDH) levels did not adversely affect the CR rate. However, 85% of the patients achieved a CR with one course of therapy, and 87% of complete responders were able to receive postremission therapy. A follow-up study testing idarubicin and HDAC yielded similar results.

Table 3
Treatment Outcome by AML Onset, CD34 and *MDR1* Expression, Cytogenetic Status, and Drug Efflux in 211 Elderly AML Patients

	Patients		Response to treatment: Complete remission		Univariate
	No.	*%*	*No.*	*%*	*p-value*
AML onset					
Secondary	50	24	12	24	0.0005
De novo	161	76	83	52	
CD34 expression					
Positive	138	68	53	38	0.0027
Negative	66	32	39	59	
MDR1 expression					
Bright/moderate (+)	102	54	35	34	0.0019
Dim (+)	33	17	15	45	
Negative	54	29	36	67	
Cytogenetic status					
Unfavorable	52	32	11	21	< 0.0001
Intermediate/favorable	112	68	62	55	
Functional drug efflux					
Positive	101	58	35	35	0.0039
Negative	74	42	43	58	

There are some clinical factors to be considered in deciding when to use HDAC in patients with newly diagnosed AML. In general, cytarabine induces a higher frequency of cerebellar toxicity that increases with increasing patient age and decreasing renal function, so use of this agent in older patients requires caution, and dose adjustments may need to be made.

The results of induction therapy for patients with AML who are older is less successful, in terms of both tolerance to the therapy and resistance of the disease. Studies from the Southwest Oncology Group have indicated that patients who are older tend to have a higher frequency of poor prognostic features, including the expression of multidrug resistance genes as well as poor-risk cytogenetics *(12,13,26)*. Table 3 shows the impact of these factors on achieving remission for patients treated with a standard regimen of cytarabine and daunorubicin. Of note, those uncommon older patients who had relatively good-prognosis cytogenetics achieved a remission with nearly the same frequency as patients who were younger, indicating that the cytogenetic features analysis of the leukemia in an older patient might have an influence on the treatment options.

3.2. Postinduction Therapy

Once remission has been achieved, further intensive therapy is required to prevent relapse. The options include the use of repeated courses of consolidation therapy, usually built around cytarabine, allogeneic transplantation from an HLA-matched family member, or, in some circumstances, unrelated donor or autologous stem cell transplantation, usually after one or more courses of consolidation therapy.

As noted above, several trials have suggested that exposure of the patient to HDAC, during either induction or consolidation, may have a beneficial impact on disease-free survival in those patients not undergoing transplantation. This has been studied most consistently by the CALGB, who conducted a trial to determine whether there was a dose-response effect for cytarabine in patients with AML and whether a beneficial effect extended to all subtypes of AML or only to specific cytogenetic subtypes *(27)*. Patients whose leukemia expressed core binding factor (CBF) abnormalities (8;21 translocation, inversion 16) showed a 50% likelihood of remaining in remission after 5 yr, compared with 32 and 15% for patients with a normal karyotype or other abnormalities, respectively. The impact of the cytarabine dose given in consolidation was most clearly seen in the CBF group, with 78% of receiving a dose of 3 g/m^2 dose still in remission. Fifty-seven percent of those patients treated at the 400 mg/m^2 dose and 16% of those treated at the 100 mg/m^2 dose remained in long-term remission, respectively. After 5 yr, among patients with a normal karyotype, the remission rates (by cytarabine dose) were 40% (3 g/m^2), 37% (400 mg/m^2), and 20% (100 mg/m^2). A dose-response effect could not be observed for patients with other cytogenetic abnormalities, with only 21% or less of this subgroup achieving long-term continuous remission.

The study demonstrated that the curative impact of cytarabine intensification after a remission induction varied significantly among cytogenetic subgroups. This treatment had its most significant impact on the prolongation of remission in those patients who had CBF abnormalities involving either t(8;21) or inversion 16 at diagnosis but not in those with other karyotypic abnormalities. Thus, for most patients with AML, older patients in particular, the use of high-dose cytarabine has a limited impact on the clinical outcome.

3.3. Autologous Stem Cell Transplantation in First CR

Many studies have been published on the use of unpurged or purged marrow for the treatment of patients with AML in first remission, usually after consolidation therapy *(1,28–37)*. Disease-free survival rates for patients in first CR have varied between 34 and 80%. Although each trial demonstrates the potential efficacy of the approach, many have been criticized for including patients characterized by widely different induction regimens, types and numbers of consolidation cycles before autologous hematopoietic cell transplantation, durations of CR before transplantation, and subsequent follow-up times. There are also differences in the stem cell product manipulation and preparative regimens. Similar to many reports of allogeneic transplantation for AML in first remission, a number of patients who otherwise would have been candidates for autologous stem cell transplantation (auto-SCT) relapsed early and were not included in the analysis of transplant results.

The Medical Research Council Leukemia Working Parties (MRC10) conducted a clinical trial to determine whether the addition of auto-SCT to intensive consolidation chemotherapy improved relapse-free survival for patients with AML in first remission *(28,38)*. After three courses of intensive consolidation therapy, bone marrow was harvested from patients who lacked a donor. These patients were then randomized to receive, after one additional course of chemotherapy, either no

further treatment or an auto-SCT following preparative treatment with cyclophosphamide and total-body irradiation. On an intent-to-treat analysis, the number of relapses was substantially lower in the group assigned to auto-SCT (37% vs 58%, $p = 0.0007$), which resulted in a superior disease-free survival rate at 7 yr (53% vs 40%, $p = 0.04$). This benefit for transplantation was seen in all cytogenetic risk groups.

In a North American study, patients in first remission with a histocompatible sibling donor were assigned to allogeneic stem cell transplantation (allo-SCT), and the remainder were randomized between auto-SCT with 4-HC-purged marrow or one course of HDAC (3 g/m^2 every 12 h for 6 d) *(30)*. The preparative regimen for both the allogeneic and autologous transplants was busulfan and cyclophosphamide. The 4-yr disease-free survival rates for chemotherapy, auto-SCT, and allo-SCT were 35, 37, and 42%, respectively. However, the impact of these different postremission therapies cannot be assessed without first considering the pretreatment characteristics of the disease. In the above-noted trial, patients were categorized into favorable, intermediate, unfavorable, and unknown cytogenetic risk groups based on their pretreatment karyotypes. Postremission survival rates varied significantly among the favorable, intermediate, and unfavorable groups, with significant evidence of interaction between the effects of treatment and cytogenetic risk status. Patients with favorable cytogenetics did significantly better after auto-SCT or allo-SCT than after chemotherapy alone, whereas patients with unfavorable cytogenetics did better following allo-SCT. These data, combined with findings by the CALGB on the dose-response curve in postremission therapy, indicates again the importance of cytogenetics in predicting the outcome of any particular postremission induction therapy.

Several groups have attempted to determine whether the addition of an immunotherapeutic strategy after achievement of minimal residual disease following auto-SCT might improve disease-free survival *(39–42)*. Interleukin-2 (IL-2), a cytokine with a broad range of antitumor effects, has been used in some patients undergoing auto-SCT for a variety of malignancies. A phase II study from the City of Hope testing high-dose IL-2 following HDAC ± idarubicin and auto-SCT was conducted in 70 patients *(43)*. The treatment strategy consisted a of post induction consolidation phase with HDAC ± idarubicin followed by granulocyte colony-stimulating factor and autologous peripheral blood stem cell collection. Patients then received total-body irradiation (12 gy), etoposide (60 mg/kg), and cyclophosphamide (75 mg/kg). IL-2 was administered upon hematologic recovery at of 9×10^6 IU/m^2 for 24 h on d 1–4 and at 1.6×10^6 IU/m^2 on d 9–18. Seventy patients (median age, 44 yr) were enrolled in the study. Twenty-nine percent had good-risk cytogenetics, 38% intermediate-risk cytogenetics, and 36% either unfavorable risk or unknown cytogenetics. Sixty patients were able to undergo consolidation therapy afterwards. With a median follow-up of 33 mo, the 2-yr probability of disease-free survival for the whole group of patients (intention-to-treat analysis) was 66% (73% for the 48 patients who actually received a auto-SCT; Figs. 1 and 2). Despite the intensive high-dose IL-2 given early after transplantation, no patient required intensive care or ventilatory support. Determining whether IL-2 has an impact on disease-free survival will require a randomized trial stratified by cytogenetic risk groups.

Taken together, these results indicate that auto-SCT in first CR after one or more courses of consolidation therapy can improve disease-free survival in selected groups of patients. Questions remain about the number and type of courses of consolidation chemotherapy, the type of preparative regimen for SCT, and the treatment of minimal residual disease after transplantation.

3.4. Allogeneic Stem Cell Transplantation in First CR

Allogeneic marrow transplantation from an HLA-matched sibling has been established as highly effective therapy for the achievement of long-term disease control in AML in first CR, with cure rates in the range of 50–60% of recipients *(44–49)*. The therapeutic effect is dependent both on the intensive preparative regimen and on the graft-versus-tumor effect resulting from the alloreactivity of the bone marrow graft. Worldwide, the most common regimens used for allogeneic transplantation have been either busulfan and cyclophosphamide, total-body irradiation and cyclophosphamide or total-body irradiation and etoposide *(47)*. Similar to the results of induction therapy and auto-SCT, there are risk factors at the time of transplantation that also predict survival. A recent analysis at the City of Hope of fractionated total-body irradiation and etoposide in 140 patients undergoing allo-SCT indicated a relapse rate of zero for the good-risk cytogenetics subgroup, contrasted with 35–40% for patients with poor risk *(50)*. Since chemotherapy alone and auto-SCT are effective against AML with good-risk cytogenetics, most patient with this risk category of disease do not undergo allo-SCT in first remission but are observed for the duration of remission and are transplanted at the time of relapse or second remission.

With the exception of patients who have AML in first CR with good-risk cytogenetics, allo-SCT is generally recommended when there is an HLA-matched sibling, particularly for patients who have either intermediate- or poor-risk cytogenetics. On the basis of published studies, it appears that the chance for maintaining disease-free survival for patients with poor-risk cytogenetics is improved with allo-SCT compared with other modalities of therapy. Numerous studies comparing allo-SCT (biologic randomization) with either chemotherapy alone or auto-SCT show decreased relapse rates and improved disease-free survival for the allo-SCT subgroup.

For patients who have poor-risk cytogenetics without a sibling donor and thus have limited options with chemotherapy or possibly auto-SCT, an unrelated donor transplant is becoming an increasingly viable option. Although these transplants are associated with enhanced risks of transplant-related complications, the poor prognosis with other modalities would favor this approach as the best means for achieving long-term disease control.

3.5. Management of Relapse

The prognosis of patients who relapse after achieving remission of their disease is somewhat dependent on the therapy they have received prior to relapse and the time from achieving remission to recurrence of disease. Recent studies have tried to

characterize the outcome based on the time to relapse as well as the use of certain agents *(51)*. These results suggest that those patients who have long remissions (i.e., >18 mo) can achieve a remission approximately half the time with a high-dose cytarabine-containing regimen, even with prior exposure to this agent. For those patients with a shorter duration of remission, the efficacy of reinduction therapy is generally decreased, and we suggest that these patients be considered for investigational therapies, as the remission rates are likely to be no worse and possibly better.

Thus, when a patient relapses, an analysis of the phenotype and karyotype of the disease, duration of remission, and the availability of a donor, either related or unrelated, often influences the decision making process about the management of relapse. For patients with a long first remission and good-risk cytogenetics, it is probably worthwhile to induce a second remission with a view to proceeding toward either auto-SCT or (if there is a sibling or unrelated donor available) allo-SCT. For those patients who have had a short remission and have a sibling donor, transplantation probably is as efficacious a strategy as an attempt at reinduction, which may or may not be successful and, indeed, may lead to organ toxicity that could preclude transplantation. The various regimens for reinduction include etoposide and mitoxantrone or the use of a program designed to overcome the acquired resistance of leukemic cells by reliance on a multidrug resistance modifier such as cyclosporin A *(52)*. The Southwest Oncology Group studied the use of cyclosporin A in combination with cytarabine and daunorubicin and showed that some patients with relapsed AML achieved a remission of significant duration with this approach *(53)*.

The management of relapse after allo-SCT poses different challenges. In this setting, both withdrawal of immunosuppression and the use of donor leukocyte infusions have been employed in attempts to stimulate the alloreactivity of the graft against the leukemia and induce remission *(54,55)*. Although this strategy has been consistently efficacious in patients with chronic myeloid leukemia, it has been less predictable in patients with AML and is somewhat dependent on the aggressiveness of the relapse. In patients with a somewhat more indolent relapse, the strategy may be more effective, as there is more time to develop an allogeneic antitumor response. For patients with a rapidly progressive disease, it is more likely that those immunostimulatory measures will need to be combined with chemotherapy to induce hypoplasia. For patients who have relapsed beyond a year following allo-SCT, withdrawal of immunosuppression and a second allogeneic transplant have been tried, with the attendant higher risks of regimen-related toxicity.

Recently, a novel agent, gemtuzumab ozogamicin (Mylotarg™), a humanized anti-CD33 monoclonal antibody conjugated with a very potent cytotoxic agent, calicheamicin, has become available for use in patients with relapsed AML *(56)*. During hematopoietic development, stem cells capable of establishing long-term multilineage hematopoiesis give rise to progenitors with diminished self-renewal capacity and different degrees of differentiation. During this process, hematopoietic cells express highly distinct cell surface antigens that, are occasionally expressed by the malignant counterpart to these cells. Some of these antigens (i.e., CD33) are present on AML cells but not on normal hematopoietic stem cells, providing the theoretical basis for use of the antibody-drug conjugate to ablate malignant myeloid and developing normal cells selectively while allowing reconstitution through the CD33-negative normal stem cells. CD33 is expressed on most, if not all, malignant precursors in patients with AML. Studies have been performed to determine whether normal hematopoiesis could be restored in patients with AML by selective ablation of cells expressing CD33 antigen. In a dose-escalation study, 40 patients with relapsed or refractory CD33-positive AML were treated with an immunoconjugate consisting of humanized anti-CD33 antibody linked to calicheamicin. Leukemia was eliminated in the blood and marrow in 20% of the 40 patients. Studies performed to evaluate the mechanism of response suggested that there was a high rate of clinical response in patients whose leukemic blasts were characterized by low dye efflux in vitro. The infusions were generally well tolerated, with a postinfusion syndrome of fever and chills being the most common side effect. These results showed that an immunoconjugate targeted to CD33 could selectively ablate malignant hematopoiesis in some patients with AML and provided the basis for phase II studies.

Several phase II studies were subsequently performed in different groups of patients with relapsed AML of different remission durations. In the aggregate, these studies have shown that approximately a third of the patients, both under and over the age of 65, can achieve a remission after a first relapse of their leukemia *(57)*. This effectiveness is comparable to that of reinduction therapy for patients with AML, particularly those with relatively short remissions. Although highly myelosuppressive, the therapy has little end-organ toxicity and has provided an option for reinduction therapy in patients with relapsed AML. It also affords a new method of cytoreduction prior to allo-SCT. The optimal use of this agent has yet to be determined, and many questions are still outstanding:

1. What is the nature of resistance to the antibody drug conjugate in patients whose leukemic cells still express CD33?
2. Can MDR-modulating agents improve the efficacy of the conjugate?
3. How does one combine gemtuzumab ozogamicin with cytarabine for induction therapy?
4. What is the role of this immunoconjugate in improving disease-free survival when it is used as consolidation therapy?

All these questions will require controlled clinical trials to determine how to use this novel agent in the most effective way.

3.6. Management of the Older Patient with AML

Given the general toxicity of remission induction therapy for AML, management of this disease in the elderly is obviously quite difficult *(58–61)*. AML is more common in older persons, yet most of the therapeutic progress in this disease has been made in patients younger than 55–60 yr, who can tolerate the intensive therapies: either chemotherapy alone or auto- or allo-SCT. In addition, as noted above, the leukemic cells of the older patient with AML often have poor-risk characteristics,

including concomitant myelodysplasia, unfavorable cytogenetics, and expression of the MDR gene. This information can be used to discuss the therapeutic or supportive care approach to an elderly patient with AML. Thus, a patient with many poor-risk features might be better served by undergoing supportive care with transfusion and intermittent antibiotics to improve the quality and probably duration of life, in contrast to patients with a relatively good prognosis, in whom a prolonged remission can be achieved. The other limitation concerns the difficulty in utilizing postremission therapy with high-dose cytarabine given that the toxicity of this medication is more evident in patients who are older and who may have concomitant abnormal renal function and other comorbid conditions.

These limitations, not only for induction but also for postremission therapy, mean that an elderly patient who does achieve a complete remission has fewer options for effective postremission antileukemic therapy. Studies are being conducted, at a variety of institutions, on the role of auto-SCT in older patients, given the optimistic results that have been obtained in several trials of AML therapy in younger patients *(62)*. The use of peripheral blood stem cells to hasten the return of hematopoiesis allows auto-SCT to be used in patients up to 70 or more yr of age, even with a radiation-based regimen.

Nonmyeloablative allo-SCT is being developed as a postremission therapeutic approach toward patients with a variety of hematologic malignancies *(63,64)*. Given the poor prognosis of AML patients over the age of 55 and the limited use of myeloablative allo-SCT in such patients, several institutions are investigating the postremission efficacy of nonmyeloablative allo-SCT in this historically poor-risk group.

4. FUTURE DIRECTIONS

A number of innovations are emerging from our improved understanding of the leukemic cell and effective treatment that may have an impact on the future therapy of patients with AML. With regard to diagnosis, the use of DNA microarray technology might allow not only better classification of patients but also a better understanding of the nature of resistance that is present at diagnosis or develops as an acquired mutation following therapy *(20)*. Such an analysis might allow us to determine, for any given genotype, who is likely to benefit from specific therapies so that in the same way we now use cytogenetic analysis to determine postremission therapy, we can exploit patterns of gene expression diagnosis to refine this decision making further.

The use of antibody-mediated cytoreduction is being explored in both induction and consolidation to determine its effectiveness in either improving the remission rate, decreasing the toxicity, or improving the effectiveness of postremission therapy in maintaining remissions in selected patients with AML. In addition, *MDR* modulation could also be utilized in postremission therapy to target the population of cells that may have survived induction therapy and have either innate or acquired resistance. More targeted therapy, as mentioned above, as well as agents that may affect angiogenesis, tyrosine kinase, and/or the genetic machinery of the leukemic cell are in development and will probably lead to more successful and less toxic therapies.

The preparative regimens for both autologous and allogeneic transplantation are also being modified. The most effective regimens, in general, include total-body irradiation, which has considerable toxicity to the gastrointestinal mucosa and may be associated with an increase in second malignancies over time. Studies are being performed with radioimmunoconjugates utilizing either anti-CD45 or anti-CD33 antibodies conjugated to a variety of radioisotopes, including iodine and yttrium *(65)*. Early studies suggest that the addition of radioimmunoconjugates to a regimen of busulfan and cyclophosphamide is an effective approach without increased toxicity. Phase II studies are under way with this approach, and phase III studies are planned to determine the relative contribution of radioimmunotherapy in preventing relapse in patients with AML. The same approach could be used in the preparative regimen for autologous transplant and would possibly not only improve the effectiveness of therapy but expand the age population for which this therapy could be utilized. In addition, improvements in supportive-care measures such as antiviral and antifungal prophylaxis, as well as the early detection and treatment of cytomegalovirus, have decreased the early morbidity and mortality of transplantation.

Nonmyeloablative transplantation, which utilizes the immunotherapeutic effect of allogeneic T-cells, is being used in older patients with AML, as noted above. Recent trials have demonstrated the greater effectiveness of peripheral blood stem cells over marrow in decreasing transplanted-related toxicity, improving hematopoietic recovery, decreasing infection, and possibly providing an improvement in the overall survival rate among patients with advanced disease. This method can now be explored in patients with first remission, given that the toxicity of the regimen is often higher than the relapse rate *(66)*.

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CHEMOTHERAPEUTIC STRATEGIES

II

ACUTE PROMYELOCYTIC LEUKEMIA

J

21 Acute Promyelocytic Leukemia

ZHU CHEN AND ZHEN-YI WANG

CONTENTS

1. INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML), identified by the French–American–British (FAB) classification as AML-M3. It accounts for approx 10–15% of all AML cases in most reports *(1)* but can be as high as 32% in some areas of China *(2)* and 46% among AML patients of American-Mexican descent *(3)*. Clinically, APL is associated with a high incidence of coagulopathy, either disseminated intravascular coagulation (DIC) or hyperfibrinolysis, which are often aggravated during chemotherapy and result in death at an early stage of treatment. Cytogenetically, APL is characterized in 95% of the cases by a balanced reciprocal translocation between chromosomes 15 and 17, t(15;17)(q22;q21), which leads to the formation of two fusion genes, promyelocytic leukemia–retinoic acid receptor α(*PML-RAR*α) or *RAR*α*-PML*, the former being considered to play a crucial role in leukemogenesis *(4)*.

Before 1986, APL was treated solely with intensive cytotoxic chemotherapy. Initial reports emphasized its poor outcome with use of standard therapy, but subsequent studies showed that the complete remission (CR) rate was similar or even superior to that achieved in other types of AML, particularly after introduction of the anthracyclines *(5)*. With daunorubicin dosages of 150–210 mg/m^2, CR rates were about 60–68%, whereas the median survival or CR duration ranged from 13 to 25 mo *(6)*. With improved supportive care and better use of chemotherapy, the outcome of APL improved still further. For example, in a randomized study carried out by the Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) group, the CR rate was 76.3% among 131 patients with newly diagnosed APL treated with idarubicin (IDA) alone, whereas that among 126 patients treated with IDA and cytarabine (ara-C) was 66%. The 5-yr event-free survival (EFS) rates were 35 and 23%, respectively, in the two groups *(7)*. The main disadvantages of chemotherapy are myelosuppression, as frequently encountered in other types of AML, and the exacerbation of hemorrhagic diathesis, leading to early death within 7–10 d of treatment. In addition, about 15–20% of APL patients show resistance to chemotherapy.

Since the introduction of all-*trans*-retinoic acid (ATRA) in 1987 as differentiation therapy *(8)* and arsenic compounds in 1992 as inducers of both apoptosis and differentiation *(9,10)*, a growing number of papers have dealt with APL treatment based on these two drugs *(11–30)*. There is now a consensus that the use of ATRA and arsenic compounds can produce not only higher CR rates through prompt amelioration of life-threatening hemorrhage, but also better disease-free survival (DFS) rates when combined with chemotherapy. Moreover, these new treatments provide a model of cancer therapy, based on the induction of differentiation and apoptosis in malignant cells, that differs markedly from conventional chemotherapy aimed at stopping cell proliferation. Hence, our discussion focuses on the treatment of APL with ATRA and arsenic compounds and the possible cellular and molecular mechanisms underlying their therapeutic effects.

2. USE OF ATRA IN DIFFERENTIATION THERAPY

2.1. In Vitro Studies on the Induction of Differentiation of Leukemic Cells

Induction of leukemic cell differentiation can be traced to 1971, when Friend demonstrated that murine erythroid leukemia (MEL) cells exposed to dimethylsulfoxide could

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differentiate to orthochromic erythroblasts *(31)*. Subsequently, other agents, such as butyric acid and hexamethylenebiacetamide (HMBA), were shown to be able to trigger MEL cell differentiation with a variety of the characteristics observed in normal hematopoiesis. Sachs *(32)* concluded in 1978 that differentiation arrest was sometimes reversible in murine myeloid leukemia cell lines incubated in a medium that contained some agents capable of inhibiting proliferation and enhancing differentiation *(32)*. Two years later, Breitman et al. *(33)* demonstrated that in vitro, retinoic acid isomers, including 13-*cis* retinoic acid (13-*cis* RA) and ATRA, could induce differentiation of the HL-60 cell line and fresh APL cells. We started to screen differentiation inducers in 1980. Using a diffusion chamber technique, we observed that a small compound, thioproline, could alleviate the inhibitory activity of mouse L6565 leukemia cells on normal hematopoiesis. We also found that thioproline and 2-amino thioproline could raise hemoglobin and cAMP content in K562 cells. In 1986, we showed that ATRA and harringtonine, an antineoplastic agent identified in China, were able to induce terminal differentiation of HL-60 cells and fresh APL cells from 14 patients *(34)*. On the basis of these results, we first tried to use ATRA in the treatment of a group of refractory APL patients and then applied the drug to newly diagnosed cases *(8,11)*.

2.2. Treatment Results with 13-*cis* RA

13-*cis* RA was first used to treat APL in 1983 by Flynn et al. *(35)*, who reported that one APL patient refractory to chemotherapy experienced an elevation of blood cells accompanied by maturation of leukemic promyelocytes after treatment with this agent, although the patient finally died of disseminated fungal infection. In 1984, Nilsson *(36)* described a patient with relapsed APL who achieved a CR with 13-*cis* RA and survived for >1 yr. Despite other encouraging reports *(37,38)*, one by Daenen et al. *(37)* in which 13-*cis* RA induced CR in a case of APL complicated by hyperfibrinolysis and Aspergillus pneumonia, a larger clinical trial performed in 1992 showed that 13-*cis* RA was generally ineffective *(39)*. Furthermore, later studies revealed that 13-*cis* RA is at least 10 times less effective than ATRA in promoting maximum differentiation of fresh APL cells in culture *(40)*. In a comparative in vitro study on NB4 cells, 9-*cis* RA, another isomer of RA, showed a higher differentiation activity than did ATRA, whereas ATRA produced better results than were obtained with 13-*cis* RA *(41)*. Now that high CR rates can be attained with ATRA treatment in large series, 13-*cis* RA is no longer used for remission induction in patients with APL.

2.3. ATRA Dosage and Pharmacokinetics

The daily total dosage of ATRA in remission induction therapy for APL is generally 45–60 mg/m^2, divided in three equal doses *(11)*. The time required to induce a CR is around 30–40 d, rarely 60 d. It was reported that a lower dosage, 25 mg/m^2/d, could yield an 80% CR rate *(42)*. With further reduction of the daily dosage to 20 mg/m^2 *(43)*, the CR rate was 92% in 27 newly diagnosed cases, similar to the 90% among 20 control patients treated with the standard dosage, and no difference was noted between the two groups in terms of the days required to attain CR, 34.4 ± 10.6 d in the former vs 37.4 ± 12.1 d in the latter.

Pharmacokinetic studies demonstrated that after oral administration of ATRA at 45 mg/m^2, the maximum plasma concentration (C_{pmax}) reached 569.1 ± 338.9 ng/mL, compared with 338.9 ± 204.7 ng/mL in the lower dose group. However, the latter concentration should be sufficient (10×10^{-7} mol/L) for inducing differentiation. The T_{peak} and $t_{1/2\beta}$ in the lower dose group were 1.7 ± 0.3 and 1.0 ± 0.2 hours respectively, very close to those after administration of a standard dose of ATRA (2.4 + 1.0 and 1.2 + 0.4 h, respectively) *(43)*.

2.4. Complete Remission Rate

In most large series of patients (>50) treated with ATRA, it has been possible to achieve CR rates of 76–95%, a higher range than reported for conventional chemotherapy (Table 1). The main reasons for these high CR rates are the reduction in early deaths caused by hemorrhage and the absence of bone marrow suppression, a common complication of cytotoxic chemotherapy. Figure 1 shows the in vivo differentiation of leukemic cells after 3 wk of ATRA treatment in an APL patient.

2.5. Postremission Treatment and Long-Term Survival

APL patients who continue to receive ATRA alone after attaining a CR on this agent will relapse within 6–12 mo because of the inability of ATRA to eradicate the leukemic clone *(11,17,18)*. It is therefore necessary to use cytotoxic agents for consolidation and maintenance therapy. It was estimated that the use of ATRA in APL could yield an approximate 2.5-fold increase in 5-yr survival rates *(23)*. In 1993, the Shanghai Cooperative Study Group on APL *(16)* summarized their 5-yr experience of the postremission treatment of APL, with a median follow-up of 36 (range, 4–60) mo. Among the 50 cases followed, 10 were treated with ATRA as a single agent, 10 with cytotoxic chemotherapy, and 30 with chemotherapy and ATRA. The median survival times of these three groups were 8, 8, and 18 mo, respectively. It is worth noting that among 30 patients receiving combination therapy, 15 were treated with sequential courses of intensive chemotherapy, intermittent ATRA and 6-mercaptopurine (6MP) + methotrexate (MTX), each given for 1 mo. The median survival time, 29 mo, was significantly better than results obtained with other protocols. Two years later, the Chinese Cooperative Study Group on APL summarized 5-yr survival probabilities (5Y-SP) for 423 APL patients with a median follow-up period of 50 mo. The mean (± SE) 5Y-SP was 0.18 ± 0.08 (n = 29) in the group treated with ATRA alone as postremission therapy, 0.51 ± 0.05 (n = 217) for standard chemotherapy, 0.68 ± 0.03 (n = 107) for chemotherapy and ATRA, and 0.71 ± 0.06 (n = 70) for the sequential intensive chemotherapy-ATRA-6MP + MTX protocol *(44)*. Although neither study was prospective nor randomized, the results were in good agreement and strongly indicated the need to incorporate cytotoxic chemotherapy into postremission treatment and the advantage of such chemotherapy in combination with ATRA over chemotherapy alone.

Importantly, these concepts have been confirmed by more recent randomized studies. For example, in 1997, Asou et al. *(21)* reported a series of 173 cases induced by ATRA and then consolidated with chemotherapy (median follow-up, 36 mo). The predicted 4-yr DFS and EFS rates were 62 and 54%, respectively, significantly higher than those of the two previ-

Table 1
CR Rate in APL Treated with ATRA*a*

Year	*Author*	*Protocol*	*No. of Patients*	*CR rate (%)*
1991	Chen et al. *(15)*	ATRA	50	94.0
1992	Chinese Cooperative Study Group *(14)*	ATRA	400	85.0
		ATRA + chemo	144	76.4
1993	Shanghai Cooperative Study Group *(16)*	ATRA	91	81.3
1994	Warrell et al. *(18)*	ATRA	79	84.8
1995	Kanamaru et al. *(20)*	ATRA ± chemo	109	89.0
1997	Asou et al. *(21)*	ATRA	62	95.2
		ATRA ± chemo	196	88.3
1997	Tallman et al. *(22)*	ATRA	172	72.1
1997	Soignet et al. *(23)*	ATRA ± chemo	95	83.2
1997	Mandelli et al. *(24)*	ATRA+ chemo	240	95.4
1999	Fenaux et al. *(30)*	ATRA ± chemo	413	92.0
1999	Burnett AK, et al. *(25)*	ATRA (short) + chemo	119	70.0
		ATRA (ext) + chemo	120	87.0

Abbreviations: CR, complete remission; ATRA, all-*trans*-retinoic acid.
[a] All series included > 50 cases.

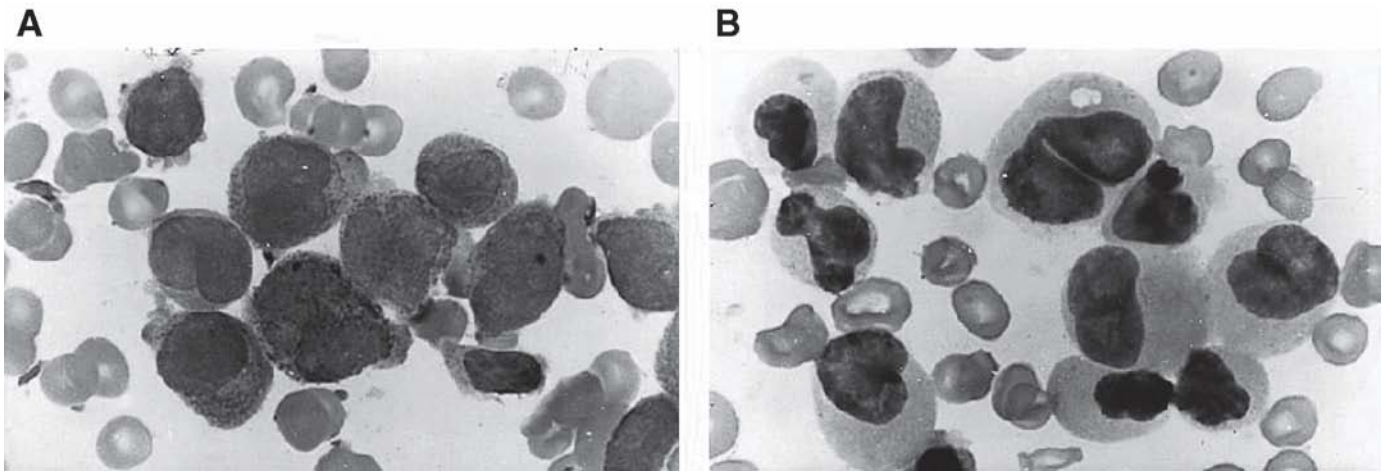

Fig. 1. Bone marrow specimen before **(A)** and 3 wk after **(B)** treatment with ATRA in an APL patient, showing differentiation of leukemic cells to more mature granulocytes.

ous protocols, designated AML-87 (40.2 and 45.3%, respectively) and AML-89 (32.2 and 31.9%, respectively) *(21)*

Tallman et al. *(45)* recently reported that among 250 patients achieving CR, the 5-yr DFS rates were 29 and 69%, respectively, for patients randomized to daunorubicin + ara-C (DA) or ATRA, regardless of the maintenance therapy given. The 5-yr overall survival rate was 44% for patients treated on the DA and 69% for those receiving ATRA. The 5-yr overall survival for patients randomized to ATRA maintenance was 60% compared with 34% for observation (Obs). If both induction and maintenance randomization are taken into account, the 5-yr DFS rates were: DA/ATRA (n = 50), 47%; DA/Obs (n = 51),10%; ATRA/ATRA (n = 48), 74%; and ATRA/Obs (n = 54), 51%.

Recently, Fenaux et al. *(30)* summarized a randomized study conducted by the European APL group for 413 patients with newly diagnosed APL. The protocol for remission induction specified ATRA followed by chemotherapy (ATRA → CT), or ATRA + CT with patients stratified by white blood cell count and age. The relapse rate at 2 yr was estimated to be 6% in the ATRA + CT group vs 16% in the ATRA → CT group (p = 0.04). The EFS rate at 2 yr was an estimated 84% in the ATRA + CT group vs 77% in the ATRA → CT group (p = 0.1). Among the 289 patients randomized to maintenance therapy, the 2-yr relapse rate was 11% in those randomized to continuous maintenance CT vs 27% in those not receiving CT (p = 0.0002) and 13% in those randomized to intermittent ATRA vs 25% in patients treated without ATRA (p = 0.02).

Taken together, these data indicate that remission induction with a regimen comprising ATRA and CT, followed by continuous CT and intermittent ATRA, may be the best strategy for achieving long-term DFS in a significant percentage of patients.

2.6. Adverse Effects and Their Management

During remission induction with ATRA, hyperleukocytosis is observed in >50–70% of the patients. A severe toxic effect called retinoic acid syndrome (RAS), often accompanied by hyperleukocytosis, can be life-threatening and hence is worthy of close attention *(46)*. RAS is characterized by fever, respiratory distress, lower extremity edema, weight gain, pleural or pericardial effusion (occasionally with hypotension) and cardiac and renal failure. Its frequency has reached 20–25% in some Western countries but is relatively low in China and Japan (7–10%). There is general agreement that addition of chemotherapy at the beginning of ATRA treatment or when the white blood cell count has increased to >5–10 × 10^9/L can ameliorate this complication. Consequently, the incidence of RAS was 5% (6/125) in a European study group and 6.4% (7/109) in the Japanese Adult Leukemia Study Group *(47)*.

Other nonspecific, common adverse effects produced by ATRA treatment include cheilosis (in as many as 70–75% of patients), headache (25–40%), arthralgia (15–60%), liver function damage (12–30%), and elevation of serum lipids (50% in Western countries). Occasionally, one may encounter hypercalcemia, erythema nodosum, marked basophilia, scrotum exfoliative dermatitis with ulcer, and even renal or cerebral thrombosis.

Usually, the adverse effects of ATRA subside after the dose is lowered and symptomatic treatment is begun. Only in rare cases should the drug be withdrawn. Nevertheless, once RAS develops, it should be treated immediately with intravenous high-dose dexamethasone (10 mg twice a d for 3–5 d or longer). With this strategy, De Botton et al. *(48)* observed that although 15% of their 413 patients experienced RAS, it was fatal in only 1.2% of the total group *(48)*. Another way to prevent or ameliorate RAS, as well as other side effects, would be to use small doses of ATRA (15–20 mg/d) *(43)*, particularly in elder patients.

2.7. *PML-RARα* and the Detection of Minimal Residual Disease

The fusion gene transcript *PML-RARα* resulting from the translocation (15;17) plays an important role in leukemogenesis, as will be discussed later. Clinically, the detection of *PML-RARα* by the reverse transcriptase polymerase chain reaction (RT-PCR) technique serves as a specific molecular marker of the disease. As described by several groups including our own *(49–51)*, this marker can be used to confirm or establish the diagnosis of APL and to predict the responsiveness of the patient to ATRA, especially when the morphologic and cytogenetic examinations conflict. Since RT-PCR analysis of *PML-RARα* is a sensitive tool with which to evaluate minimal residual disease (MRD), it can also be used to prejudge the relapse of the disease and to guide continuation therapy. For example, Huang et al. *(49)* described the results of detecting the *PML-RARα* transcript in 97 APL patients. Among 12 cases analyzed before and after CR induction by ATRA, 9 remained positive by RT-PCR assay, confirming that ATRA is not sufficient to induce a molecular remission. Among 52 APL patients followed up for 3–72 mo after ATRA-induced CR and consolidation therapy, 11 had a positive RT-PCR test. Whereas all patients with a negative RT-PCR result remained in good clinical remission, 5 of the 11 cases with a positive test relapsed within 1–6 mo.

2.8. Treatment of Relapse

As mentioned above, if an ATRA-induced CR is not consolidated with cytotoxic agents, it usually lasts only 6–12 mo. Pharmacokinetically, prolonged use of ATRA causes a decreasing maximum plasma concentration of the drug *(46)*. There can be several reasons for drug resistance to occur, including the metabolic change of ATRA caused by induced expression of CRABP II and cytochrome P450, the induction of P170, and the selection of leukemic clones with mutation in the ligand binding domain of *PML-RARα (4,46,47,52)*. It is therefore necessary to administer cytotoxic chemotherapy to eradicate the leukemic clone. However, even with the best postremission treatment, relapse will still develop in 30–40% of patients within 5 yr after CR induction. Once relapse occurs, only a small fraction of the patients respond to ATRA/chemotherapy. In a retrospective analysis of 153 relapsed cases performed by the Chinese Cooperative Study Group on APL, the second remission rate was 30.1% overall upon treatment with chemotherapy combined or not combined with ATRA *(44)*. It was expected that 9-*cis* RA could be an effective agent in treating relapsed APL, because it possesses high affinity for RA receptors, particularly the retinoid X receptor (RXR). Nevertheless, when tested in a clinical trial, it failed to reverse acquired retinoid resistance *(53)*. A retinoid analog designated Am80 was reported to be a promising compound for relapsed APL, in that a second CR rate of 54% was achieved in a series of 24 patients *(54)*. However, more studies are needed to evaluate the therapeutic value of this analog further. Fortunately, the use of an ancient class of drugs, arsenic compounds, has proved to be highly effective in the treatment of relapsed APL.

3. TREATMENT OF APL WITH ARSENIC COMPOUNDS

3.1. Historical Review

By 1865, arsenic compounds had already been used for the treatment of chronic myeloid leukemia, in the form of potassium arsenite (Fowler's solution) *(55)*. It was abandoned in the mid-20th century because of chronic toxicity and, more importantly, because of the discovery of chemotherapeutic agents such as busulfan. Before the antibiotic era, several organic arsenic compounds were also used clinically as antispirochaeta agents, but most of them were replaced by more effective and less toxic drugs. Nowadays, only melarsoprol remains an important agent for the treatment of African trypanosomiasis *(27,56)*. In traditional Chinese medicine, two arsenic compounds have been used for >500 yr. One is *Pishuang*, or white arsenic, containing essentially arsenic trioxide (As_2O_3). It was recorded in the *Compendium of Materia Medica* (edited by Li Shizhen, 1518–1593) and until

Table 2
Arsenic Compound-induced CR Rate in APL

Year	*Author*	*Arsenic compound*	*Status of the disease*	*No. of Patients*	*CR rate (%)*
1992	Sun et al. *(9)*	Ailing-1 (Al-1)	De novo + relapsed	32	65.6
1995	Huang et al. *(58)*	Composite Indigo Naturalis tablets	De novo + relapsed	60	98.0
1996	Zhang et al. *(26)*	As_2O_3	De novo	30	73.3
			Relapsed	42	52.4
1996	Chen et al. *(56)*	As_2O_3	Relapsed	16	93.7
1998	Soignet et al. *(28)*	As_2O_3	Relapsed + refractory	12	92.0
1999	Niu et al. *(29)*	As_2O_3	De novo	11	72.7
			Relapsed	47	85.1
1999	Soignet et al. *(57)*	As_2O_3	Relapsed + refractory	40	85.0

Abbreviations: CR, complete remission.

recently was administered in clinics for the treatment of asthma, certain skin diseases, wound healing, and some surgical conditions. Another compound is *Xiong-huang*, or realgar compound, containing arsenic sulfide. It is usually administered in the form of *Bezoar antitoxic pill*, which consists of realgar, bezoar, and *Baphicacanthus cusia*. The pill is given in clinics for the treatment of sore throat and oral or other infections.

In early 1970s, a group of clinical investigators from Harbin Medical University in Northeast China began to use white arsenic for the treatment of malignancies, based on the traditional Chinese medical principle of "using toxic agents to fight against something toxic." After large-scale clinical screening, several human cancers were identified as suitable targets, including esophageal carcinoma, lymphoma, and leukemia. In 1992, the same group described for the first time the intravenous administration of Ailin-1 (anticancer-1) solution, containing 1% As_2O_3 and a trace amount of mercury chloride, to patients with APL. Of 32 cases treated, the solution induced CRs in 21 (65.6%). The 5-yr survival rate was 50% and the 10-yr survival rate 18.8% *(9)*. In 1996, two groups in China reported on the results obtained with pure 1% As_2O_3 solution. In one study, 22 of 30 patients (73.3%) with *de novo* APL and 22 of 42 (52.4%) with relapsed or refractory APL achieved CR *(26)*. In another clinical trial performed only in relapsed APL, 15 of 16 patients (93.8%) attained CR with a daily dose of 10 mg/d for 28–54 d *(56)*. These results have been confirmed by other institutions in Western countries (Table 2) *(28,57)*.

Realgar also seems to have efficacy as remission induction therapy for APL. In 1995, Huang et al. *(58)* reported that among 60 APL patients, including 43 newly diagnosed and 14 refractory cases, Composite Indigo Naturalis tablets (containing realgar, *Baphicacanthus cusia*, *Radix salviae miltiorrhizae*, and *Radix pseudostellariae*) with or without mild chemotherapy, produced a CR rate of 98%. Recently, Lu et al. *(59)* performed a study of pure tetra-arsenic tetrasulfide (As_4S_4) for the treatment of APL among 100 patients. Of the 93 who were evaluable, 79 (84.9%) achieved CR, with an acturial 3-yr EFS rate of 84.4% *(59)*.

3.2. Dosage and Pharmacokinetics

According to experience at the Shanghai Institute of Hematology, the standard dose of As_2O_3 is 10 mg/d for adults (0.16 mg/kg/d for all ages), so that 10 mL of a 1% solution is diluted into 250–500 mL of 5% dextrose in normal saline and infused intravenously over 2–4 h. A course of 28–54 d (median, 31 d) is necessary to induce a CR. Accordingly, the total dose of the drug is between 280 and 540 mg *(27,29)*. Recently, a lower daily dose of 5 mg for adults (0.08 mg/kg/d) was tried in the Shanghai Institute of Hematology. Interestingly, the CR rate (23/25, or 92%) was similar to that attained with a standard dose.

Pharmacokinetic studies showed that after infusion of 10 mg As_2O_3, the plasma arsenic concentration rapidly reached the peak level, with a mean C_{pmax} of 6.85 μmol/L (range, 5.54–7.30 μmol/L), $t_{1/2\alpha}$ of 0.89 ± 0.029 h, and $t_{1/2\beta}$ of 12.13 ± 3.31 h (n = 8 patients) *(27)*. The pharmacokinetic features did not change, even when determined 30 d after CR induction, indicating that early tolerance did not occur, in contrast to findings with ATRA. Measurement of urinary arsenic content was slightly increased during drug administration. The total amount of arsenic excreted daily in urine accounted for approximately 1–8% of the total daily dose. The urinary excretion of arsenic persisted after suspension of the drug, although the amount excreted slightly declined. Arsenic content in both hair and nails gradually increased over treatment, with the peak level of arsenic reaching 2.5–2.7 μg/g of tissue when CR was induced, or five to seven times higher than before treatment (0.35–0.40 μg/g). However, the hair and nail arsenic content was significantly decreased during the treatment interval.

Composite Indigo Naturalis tablets are administered per os three times a d (total daily dose, 15 tablets). The dose is increased progressively to 30 tablets/d until CR is attained. Usually the duration of a course exceeds 1 mo. Glycosteroids or mild chemotherapy can be administered simultaneously. As_4S_4 is given initially at a dose of 0.5 g and escalated to 1.0 g/d per os until CR is attained. One course usually extends over 2–4 wk, with intervals of 2–3 wk between courses. So far, pharmacokinetic data are not available.

3.3. Adverse Effects

As_2O_3-related toxic effects include skin reactions (25–30% of patients), such as dryness, itching, erythematous changes, and pigmentation; headache during the infusion of the drug (7%); arthralgia or muscle pain (14%); gastrointestinal disturbances (21–27%), such as vomiting, nausea, and diarrhea; peripheral neuropathy with dysesthesia (25%); and liver function damage with elevation of AST, ALT, alkaline phosphatase, GGT, or total bilirubin (7–30%) *(27–29)*. From 7 to 10% of the patients show electrocardiographic (ECG) changes with a low-flat T-wave, sinusal tachycardia and a first-degree A-V block. On rare occasions patients may require insertion of a pacemaker owing to a second-degree heart block. As with ATRA treatment, 50–60% of the patients can develop hyperleukocytosis, with white blood cell counts as high as $150–170 \times 10^9$/L. Among some patients, symptoms similar to those of RAS with respiratory distress may appear, necessitating emergency assistance. Other less frequent adverse effects are enlargement of the salivary gland, enlarged thyroid gland without hyperthyroidism, toothache, oral ulcer, and gingival or nose bleeding.

In general, the toxic effects are mild and can subside with reduction of the dose or with symptomatic treatment. However, RAS-like symptoms must be treated with dexamethasone and the addition of chemotherapy. Comparisons of toxic effects produced by As_2O_3 and arsenic sulfide are not yet available. However, the use of As_2O_3 in *de novo* APL should be undertaken with caution, since severe toxic effects leading to hepatic failure were recently reported *(29)*. Under these circumstance, withdrawal of the arsenic compound is mandatory, with prompt introduction of appropriate supportive care.

3.4. Postremission Treatment and Long-Term Survival

Although As_2O_3 can yield a high CR rate in relapsed patients, maintaining the CRs remains a challenge. Generally speaking, it is necessary to give two or three courses of arsenic treatment as consolidation after CR induction. Any of three strategies can be selected for subsequent therapy: (1) chemotherapy alone (if possible, use drugs to which patients were not previously exposed); (2) continuation of the arsenic treatment; or (3) a combination of chemotherapy and an arsenic compound.

A recent study conducted at the Shanghai Institute of Hematology addressed the issue of postremission treatment in a group of relapsed APL patients reinduced with As_2O_3 *(29)*. In the chemotherapy group, patients were treated with daunorubicin and ara-C (DA protocol) or mitoxantrone and ara-C (MA protocol), one course every 2 mo in the first year, every 3 mo in the second, and every 4 mo in the third. A second group of patients received As_2O_3 continuously at a daily dose of 10 mg for 28–30 d every 2–3 mo during the first yr and for approximately 7–14 d every 2 mo over the second and third yr. A third group was treated with a chemotherapy and As_2O_3 combination: chemotherapy was the same as that for the first group, and As_2O_3 was the same as that for the second group. Among 33 patients followed for 7–48 mo, the estimated DFS rates at first and second yr were 63.6% and 41.1%, respectively, and the actual median DFS rate was 17 mo. The DFS was much better in the group treated with As_2O_3 and chemotherapy than in patients treated with As_2O_3 only (2/11 vs 12/18 relapses, $p < 0.01$). Other factors that influenced the outcome were the number of relapses and white blood cell count at relapse. If ATRA is withdrawn for >6 mo, its addition could be beneficial, particularly when the response to arsenic is delayed or not very striking. It has been proposed that if APL patients with high risk of relapse can be identified, they might benefit from incorporation of As_2O_3 into the postremission treatment. This strategy might be one way to increase the 5-yr DFS rate in APL still further.

3.5. Correlation between *PML-RARα* Expression and Response to As_2O_3

According to a recent study, the presence of t(15;17) together with a positive RT-PCR result for *PML-RARα* indicates a favorable response to arsenic treatment *(29)*. By contrast, in a few relapsed cases with evidence of clonal evolution (either the loss of *PML-RARα* expression or the appearance of fusion genes other than *PML-RARα*) there was no response to As_2O_3. The significance of RT-PCR data in relapsed patients reinduced with As_2O_3 remains uncertain. Soignet et al. *(28)* reported that after two courses of As_2O_3 therapy, 8 of 11 patients lost RT-PCR positivity for *PML-RARα*. In our recent study, this fusion gene was detected in 14 of 15 cases after arsenic induction of CR *(29)*, a result that was recently confirmed by quantitative real-time PCR (B. W. Gu, J. Hu, S. J. Chen, and Z. Chen, unpublished data). This discrepancy may reflect the higher sensitivity of the RT-PCR technique used in our study. Whatever the explanation, we prefer to believe that, as seen with ATRA treatment, remission induction with an arsenic compound cannot eradicate the leukemic clone in most patients, although long-term use of the compound could indeed lead to molecular remission in a few patients. This implies that maintenance of CRs with chemotherapy or ATRA should be beneficial if combined with As_2O_3.

3.6. Changes in Coagulopathy During Treatment with Both ATRA and As_2O_3

One advantage of both ATRA and As_2O_3 is their ability to correct the bleeding syndrome rapidly in APL, in contrast to cytotoxic chemotherapy, which causes early death owing to hemorrhage in about 10–20% of patients. The mechanism underlying the coagulopathy occurring in APL is complex *(60)*. Alterations of laboratory coagulation tests and the clinical picture can reflect DIC or hyperfibrinolysis; hypofibrino-genemia; prolonged prothrombin and thrombin times; increase of fibrinogen–fibrin degradation products (FDP); elevations of the thrombin–antithrombin complex (TAT), prothrombin fragments 1 + 2, or fibrinopeptide A; low plasminogen; elevated tissue plasminogen activator (t-PA) and urokinase (u)-PA; a low level of α_2-antiplasmin in plasma; and thrombocytopenia *(60)*. The initial causes of these abnormalities have been ascribed to the procoagulant activities and to some enzymes expressed and/or released by APL cells, including tissue factor (TF) *(61)*. In a recent study of the changes in plasma levels of hemostatic parameters and TF in APL patients during ATRA and As_2O_3 treatment *(62)*, both TF antigen and procoagulant activity declined remarkably in bone marrow mononuclear cells 7 d after ATRA or arsenic

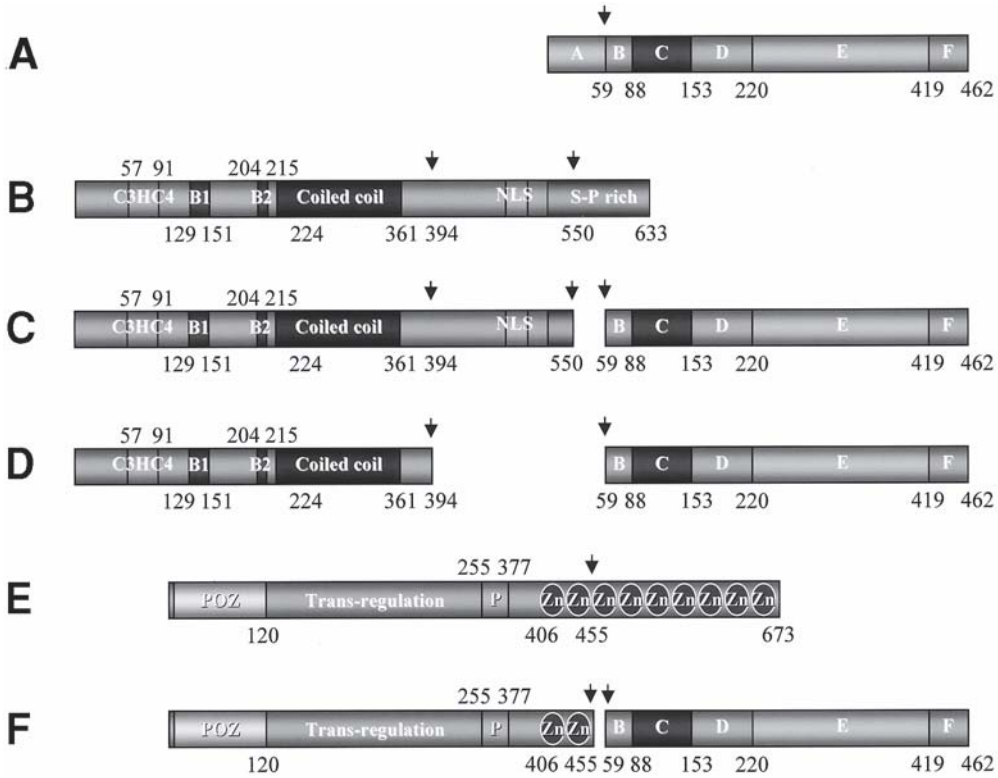

Fig. 2. Schematic representation of proteins involved in t(15;17)(q23;q21) and t(11;17)(q23;q21). **(A)** RARα, **(B)** PML, **(C)** Long-type (PML/RARα-L), **(D)** short-type (PML/RARα-S) isoforms of PML-RARα, **(E)** PLZF, and **(F)** PLZF-RARα. Arrows indicate chromosomal breakpoints and sites of fusions. Please see text for the details of structure/function domains of each gene.

treatment, becoming undetectable 14 d after therapy. Fibrinogen and α_2-antiplasmin activity in plasma, which were low at presentation, increased progressively during the treatment. By contrast, levels of soluble fibrin monomer, plasminogen activity, D-dimer, and TF antigen were elevated before treatment but gradually decreased during the administration of ATRA and As_2O_3. Interestingly, both ATRA and As_2O_3 can downregulate TF on APL cells through transcriptional regulation. It was also noted that ATRA treatment downregulated the APL cellular expresson of cathepsin G, a serine protease able to cleave fibrinogen and plasminogen activator inhibitor type 1 (PAI-l) *(62)*. Retinoids have been shown to enhance t-PA synthesis by endothelium cells *(60)*, but these alterations have not yet been studied in patients treated with arsenic compounds. It is noteworthy that an in vitro study of NB4 cells revealed upregulation of cellular TF and membrane procoagulant activity upon exposure of the cells to daunorubicin, explaining at least partly the mechanism by which antineoplastic drugs aggravate the coagulopathy *(62)*.

4. MECHANISMS OF ACTION OF ATRA AND AS_2O_3 IN APL

4.1. Physiology of the RA Pathway

The specific effects of both ATRA and As_2O_3 on APL cells suggest a link between the mechanisms of action of these agents and those underlying the leukemogenesis. Indeed, over the last decade, molecular studies identified the disruption of the *RAR*α gene and its fusion to partner genes as the major event in APL pathogenesis *(4,46,63,64)*. It is now well known that RA isomers are a group of active metabolites of vitamin A and that their physiologic effects are exerted through two families of RA receptors: RAR and RXR *(65,66)*. RARs can bind both ATRA and 9-*cis* RA, whereas RXRs bind only 9-*cis* RA with high affinity. Each receptor family has three members (*RAR*α, -β, and -γ, and *RXR*α, -β, and -γ) each encoded by a different gene. Each member has a number of isoforms because of the alternative use of the promoters of the gene.

Both RARs and RXRs share homology with other members of the nuclear hormone receptor superfamily, such as steroid hormone receptors, vitamin D receptors, and thyroid hormone receptors. As hormone-inducible transcription factors, these receptors possess six domains (A–F; Fig. 2A): A/B for ligand-independent transactivation; C with two zinc fingers for DNA binding; D, a hinge region; and E, which is responsible for dimerization with RXR, ligand binding, ligand-dependent transactivation, and association with corepressor or coactivator complexes. The function of the F domain is still unknown. RXR can form a heterodimer not only with RAR but also with other members of the nuclear receptor family, including some "orphan" receptors (receptors without known ligand). The RAR/RXR heterodimer is the active form of the RAR, which binds to specific DNA sequences designated as the retinoic acid response element (RARE) in the promotor regions of target genes and regulates the transcriptional expression of these genes. Because of the great multiplicity of receptors resulting from heterodimerization between distinct RARs and RXRs and from the differential use of isoforms of each family member, extremely diverse biologic responses can be generated.

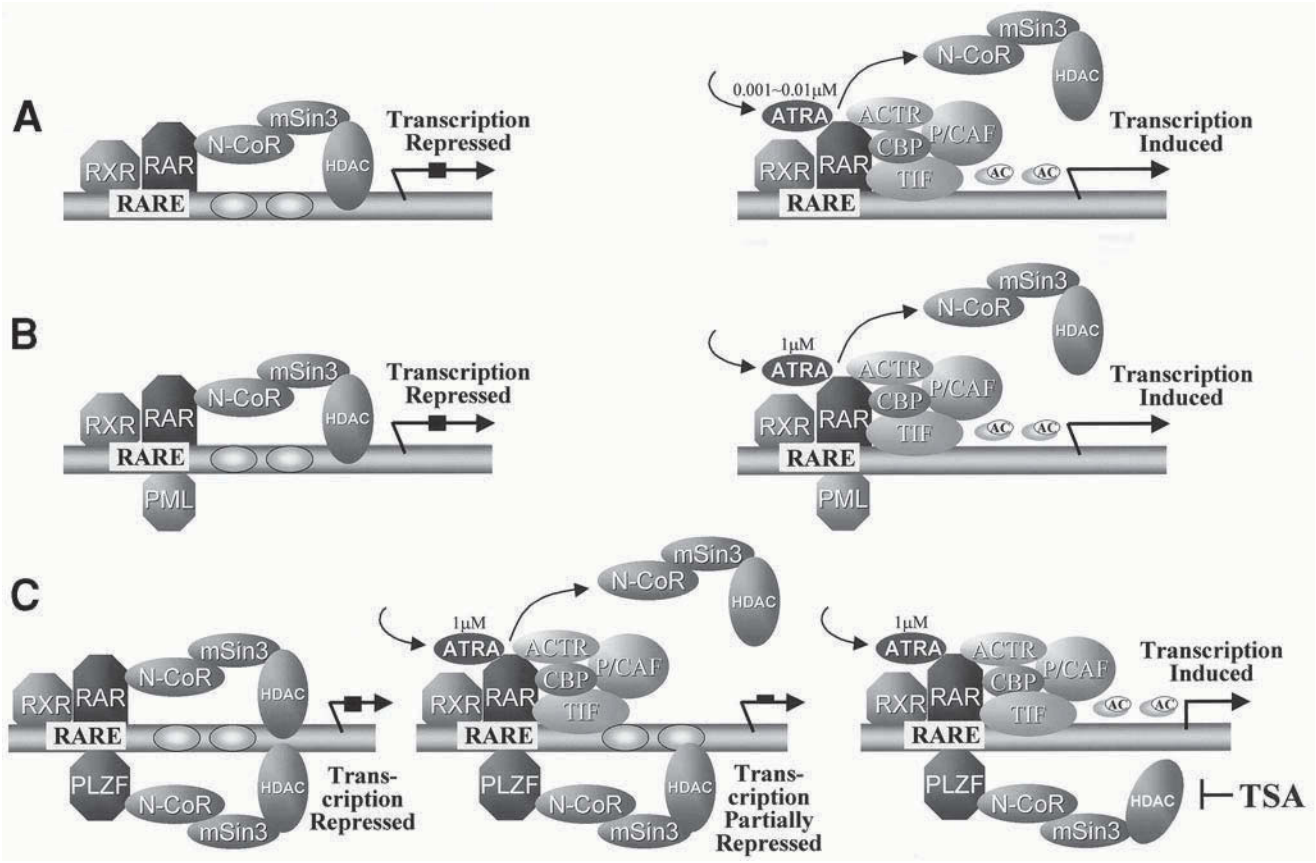

Fig. 3. Potential mechanisms underlying the pathogenesis of APL and all-trans-retinoic acid (ATRA) differentiation therapy. (**A**) ATRA remodeling of wild-type receptor heterodimer and target gene transcription: (left) transcription is repressed in the absence of ligand; (right) under physiologic concentrations of ATRA, corepressor (CoR) is released from and coactivator (CoA) binds RARα/RXR, resulting in activation of transcription. (**B**) Modulation of PML/RARα by pharmacologic concentrations of ATRA: (left) transcription repressed in the absence or under physiologic concentrations of ligand; (right) 1 μM ATRA leads to the release of CoR and recruitment of CoA, leading to transcription of target genes. (**C**) Relative resistance of PLZF/RARα-associated APL to ATRA: (left) transcription repressed in the absence or under physiologic concentrations of ligand; note the two binding sites for CoR; (middle) pharmacologic concentration of ATRA is not sufficient to release CoR, because the POZ/BTB-CoR binding is insensitive to ATRA modulation; (right) histone deacetylase inhibitors such as trichostatin A (TSA) in combination with ATRA can relieve the transcriptional repression. AC, acetylated histones; ACTR, receptor co-activator; CBP, CREB-binding protein; HDAC, histone deaceytlase; N-CoR, nuclear receptor corepressor; P/CAF, P30d/CBP-associated factor; RAP, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; TIF, transcriptional intermediary factor.

Recently, new progress with regard to the molecular regulation of RA signaling has been made, thanks to the discovery of corepressor (CoR) and coactivator (CoA) complexes *(66,67)*. CoR is composed of at least three types of proteins, the nuclear receptor corepressor (N-CoR) or silencing mediator of retinoid and thyroid receptor (SMRT), mSin3A or mSin3B, and histone deacetylase (HDAC). CoA is also a multiprotein complex, containing the transcriptional intermediary factor-1 (TIF-1), the CREB binding protein (CBP)/adenoviral E1A-associated protein p300(P300), P300/CBP-associated factor (P/CAF), the nuclear receptor coactivator ACTR, and the nuclear receptor coactivator-1 (NcoA-1), also called steroid hormone receptor coactivator-1 (SRC-1), or NcoA-2, also called TIF-2. P300/CBP, P/CAF, and ACTR possess histone acetylase (HAT) activity. In the absence of the ligand, RAR/RXR is associated with CoR, which keeps the chromatin structure of the target genes in a repressed state through deacetylation of histones. Upon binding of RA, CoR is dissociated from RAR/RXR, and CoA binds to the receptor heterodimer. CoA not only opens the chromatin structure by acetylating nucleosomes but also recruits the basic transcription complex to the target genes so that their transcriptional expression is activated (Fig. 3A).

The physiologic importance of RA regulatory pathways in vertebrate ontogenesis has been demonstrated in knockout animal models. Mouse double mutants for *RARα/RARβ*, *RARβ/RARγ* or *RARα/RARγ* all died either in fetal life or shortly after birth, with a variety of congenital abnormalities similar to those reported in vitamin A-deficient fetuses *(68,69)*. RA regulatory pathways have also been shown to play a central role in the development of hematopoiesis. Suppression of the endogenous RAR activity by a dominant negative RAR construct could block neutrophil differentiation at the promyelocyte stage in the mouse FDCP mix A4 cell model *(70)*.

4.2. APL Pathogenesis

4.2.1. APL with t(15;17) (q22;q21) and *PML-RAR*α

In the classical translocation, the *RAR*α gene is fused with the *PML* gene to form the *PML-RAR*α chimera, which is expressed in all APL patients with t(15;17) *(71,72)*. The reciprocal fusion, *RAR*α*-PML*, is expressed in only 60–70% of the patients and therefore is not considered as key player in leukemogenesis. A large body of evidence shows that *PML-RAR*α encodes a chimeric receptor with abnormal functions, compared with the wild-type proteins expressed by the normal alleles present in the same cell *(4,63,64)*. PML is a nuclear protein that contains, from its N to C terminus, a proline-rich sequence, a cysteine-rich region [including three zinc-finger-like structures, namely, a RING (really interesting new gene) domain and two B-box zinc fingers] responsible for nuclear body localization, a coiled-coil region for homo/heterodimerization, a nuclear localization signal (NLS), and a serine/proline-rich domain (Fig. 2B). Topologically, PML is a major component of an organelle called the PML nuclear body or oncogenic domains (PODs), which are present as large nuclear speckles (10–20 in number) in most of the cells investigated *(73,74)*. PML functions as a growth inhibitor and apoptosis agonist and may play an important role in the regulation of transcription *(75,76)*. The major functional domains of *PML* (i.e., cysteine-rich region and coiled-coil domain) are retained in the *PML-RAR*α fusion gene in either "long" or "short" isoforms caused by distinct breaks in chromosome 15 within the *PML* locus *(77)* (Fig. 2C and D). PML-RARα can form a heterodimer with wild-type PML and thereby disrupt POD speckles into hundreds of micropunctates *(73,74)*. The dominant negative effect of PML-RARα on PML may allow the cells to acquire a growth advantage and resistance to apoptosis.

On the other hand, PML-RARα heterodimerizes with RXR via the E domain of the RARα moiety, so that this essential partner of RARα is sequestered *(78)*. As a result, the RARα/RXR pathway necessary for granulocytic differentiation is abrogated. Moreover, PML-RARα forms a homodimer through the coiled-coil motif and competes with RARα for binding to RARE of target genes. In contrast to the RAR/RXR heterodimer, the PML-RARα homodimer interacts with CoR with a much higher affinity, which mediates the transcriptional repression under physiologic concentration of RA and blocks the cell differentiation program *(79,80)*.

4.2.2. Variant Fusion Genes in APL and Their Biologic Significance

In 1992, a variant chromosomal translocation [t(11;17)(q23;q21)] was identified in a Chinese patient diagnosed with APL *(81)*. Since then, at least eight confirmed APL cases with t(11;17)(q23;q21) have been reported in the literature *(64)*. Morphologically, the variant form of APL shows some differences from t(15;17)-positive APL, with a predominance of cells with regular nuclei, either with many granules or more rarely with few granules, and an increased number of Pelger-like cells *(82)*. Another feature of t(11;17)(q23;q21) leukemia is that, in contrast to the great majority of APL cases with t(15;17), the affected cells are not sensitive to ATRA. For these two reasons, t(11;17)(q23;q21)-positive leukemia has been recently considered by some authors as a distinct clinical syndrome *(64)*. Molecular cloning of the t(11;17) (q23;q21) translocation has revealed that a gene called the promyelocytic leukemia zinc finger (*PLZF*), normally located on chromosome 11q23, is translocated with *RAR*α. PLZF belongs to a zinc-finger protein family termed zinc-finger protein with interaction domain (ZID), characterized by the presence of the poxvirus and zinc finger (POZ) or Broad Complex, tramtrack, Bric-a-Brac (BTB) domain at the N-terminus and nine Krüpple-like C_2H_2 zinc fingers at the C-terminus (Fig. 2E).

The fusion between *PLZF* and *RAR*α results in both *PLZF-RAR*α and *RAR*α*-PLZF* chimeric genes, suggesting that both may play a role in leukemogenesis (Fig. 2F) *(64)*. The PLZF-RARα protein contains the POZ/BTB and the first two zinc fingers of PLZF fused to B-F domains of RARα whereas RARα-PLZFis formed by the A domain of RARα and the third to ninth zinc fingers from PLZF.

Functional analysis has revealed that PLZF-RARα heterodimerizes with RXR and PLZF and therefore may block both RARα/RXR and PLZF regulatory pathways *(83)*. Owing to the POZ/BTB interaction, PLZF-RARα can also form homodimers, which show slightly different RARE binding behaviors than do those formed by PML-RARα *(83)*. More importantly, compared with PML-RARα, PLZF-RARα has a much tighter association with CoR. In fact, PLZF/RARα has two binding sites for CoR, one on the E domain of *RAR*α and another within the POZ/BTB. Although the first binding site can be regulated by ATRA, the second is insensitive to the modulation of ligand. Hence, the chromatin structure remains in repressed status, even when exposed to pharmacologic concentrations of ATRA. This biochemical property may underlie clinical resistance to ATRA in patients with expression of *PLZF-RAR*α *(79,80,84,85)*. On the other hand, some studies on RARα-PLZF have suggested that it may promote expression of the Cycline A gene *(86)* and may block the granulocyte colony-stimulating factor (G-CSF) pathway (T. Hoang, personal communication).

Over the last few years, other variant chromosomal translocations with resultant fusion genes were reported in a few APL patients, including t(5;17)(q35;q21) with the nucleophosmin (*NPM*) gene fused to *RAR*α *(87)*, t(11;17)(q13;q21) with formation of the nuclear mitotic apparatus *(NuMA)-RAR*α chimeric gene *(88)*, and the *STAT5b-RAR*α fusion caused by a duplication of 17q21.3-q23, causing interstitial chromosomal rearrangement between the *STAT5b* locus and the *RAR*α gene *(89)*. Of note, leukemic cells from patients with t(5;17)(q35;q21)/*NPM-RAR*α or t(11;17)(q13;q21)/*NuMA-RAR*α were sensitive to ATRA, whereas those from a patient with *STAT5b-RAR*α failed to respond to this drug *(87–89)*. The variant chromosomal translocations, although found in very few cases, may be of biologic significance, since they provide new models for understanding the pathogenesis of APL and the molecular basis for ATRA/As_2O_3 differentiation/apoptosis therapy. Table 3 compares the major clinical and biologic characteristics of different fusion genes so far identified in APL, including responsiveness to ATRA and As_2O_3.

4.2.3. Leukemogenic Power of Chimeric RA Receptors

Definitive evidence for the transforming ability of APL fusion genes comes from transgenic animal models. *PML-*

Table 3
Characteristics of Five Subtypes of APL

Characteristic	*t(15;17) (q22;q21)*	*t(11;17) (q23;q21)*	*t(11;17) (q13;q21)*	*t(5;17) (q35;q21)*	*dup(17) (q21.3q23)*
Frequency	>95%	1–2%	Rare	Rare	Rare
Fusion gene (frequency)	*PML-RARα* 100%	*PLZF-RARα* 100%	*NuMA-RARα* Yes	*NPM-RARα* Yes	*STAT5b-RARα* Yes
(frequency)	*RARα-PML* 60–70%	*RARα-PLZF* 100%	*RARα-NuMA* No	*RARα-NPM* Yes	*RARα-STAT5b* No
Nuclear localization	In 100 micro-speckles, may be localized in cytoplasm	Localized in microspeckles	Microspeckled pattern	Sheet-like aggregation	Microspeckled pattern, may be localized in cytoplasm
Transgenic animal models	Several, one closest to human APL	Chronic myeloloid leukemia-like phenotype	Unknown	From typical APL to chronic myeloid leukemia	Unknown
Response to					
ATRA	Good	No, may respond to ATRA + G-CSF	Good	Yes	No
Chemo	Good	No, may respond to chemo +ATRA			
Arsenic	Good, with degradation of PML-RARα	No, absence of PLZF/RARα degradation	Unknown	Unknown	Unknown

Abbreviations: RARα, retinoic acid receptor α; ATRA, all-*trans*-retinoic acid; G-CSF, granulocyte colony-stimulating factor.

RARα driven by either hMRP8 or hCG promoters, induced an APL-like leukemia in mice at approximately 1 yr after birth *(90,91)*. When transplanted into mice of the same strain, the APL-like cells generated the same leukemia. As in human APL with *PML-RARα*, CRs could be induced in the mice with either ATRA or As_2O_3. By contrast, transgenic mice with *hCG-PLZF-RARα* developed leukemia much earlier in life, at about 3–12 mo after birth *(85,91)*. These leukemias resembled chronic myeloid leukemia, with increased number of promyelocytes in the bone marrow and were insensitive to ATRA. *NPM-RARα* transgenic mice developed either typical APL or chronic myeloid leukemia-like leukemia 1 yr after birth, which seemed to respond to the differentiation-inducing effect of ATRA *(85)*.

4.3. Mechanisms Underlying ATRA Differentiation Therapy

4.3.1. Modulation of PODs and Chimeric RA Receptors

An important finding in understanding the action of ATRA was that the treatment of APL cells in vitro or in vivo with this agent induces relocalization of the PML and restores the normal structure of PODs *(73,74)*. Next, it was reported that the binding of ATRA to receptors could cause degradation of PML-RARα *(92–94)*, a process that seems to be biphasic, with a rapid decrease of the fusion protein within 1 h, followed in 12 h by a second step characterized by the appearance of a 90-kDa cleavage product. Both the proteasome-ubiquitin system and the caspase system have been suggested to be involved in the degradation of PML-RARα.

A recent study demonstrated more clearly two proteolytic pathways for RA receptors: one is the caspase-mediated cleavage of fusion protein, and the second is a proteosome-dependent degradation of both PML-RARα and wild-type RARα as well as RXRα *(95)*. The catabolism of *RARα* seems to require heterodimerization with RXRα, DNA binding, and the ligand-dependent activation domain. However, the significance of PML-RARα/RARα degradation in ATRA-induced APL cell differentiation remains controversial *(94)*. By contrast, many studies have suggested that the modulation of CoR binding of the receptors plays a key role in mediating differentiation. It is well established that a physiologic concentration (0.01 μ*M*) of ATRA is sufficient to dissociate CoR from wild-type RARα-RXRα and to recruit CoA for transcriptional activation. Although PML-RARα and NPM-RARα are less sensitive to ligand-induced modulation, pharmacologic concentrations (0.1–1 μ*M*) of the drug result in the release of CoR from these fusion receptors, subsequent recruitment of CoA, and conversion of PML-RARα and NPM-RARα from transcription repressors to transcription activators (Fig. 3B) *(79,80,84,85,96)*. It is worth noting that even in the presence of 10 μ*M* ATRA, PLZF-RARα remains associated with CoR (Fig. 3C), because of the insensitivity of the POZ/BTB-CoR interaction to ATRA modulation.

These important findings explain why APL patients with *PML-RARα* and *NPM-RARα* respond to ATRA differentiation therapy, whereas patients with *PLZF-RARα* are insensitive to the drug. Interestingly, the HDAC inhibitor trichostatin A (TSA) or sodium butyrate can overcome the transcriptional

Table 4
Examples of Genes Upregulated by ATRA in the Differentiation of NB4 Cells

Transcription factors and general DNA binding proteins	
Myeloid cell nuclear differentiation antigen	
DNA-binding protein inhibitor ID-2	
Nuclear receptor coactivator ACTR	
C/EBPε	
Cytokines, chemokines and growth factors	
Monocyte chemotactic protein 1	
Interleukin-8	
Interleukin-1β	
Signal transduction modulators and effectors	
Protein kinase C type β1	
Calmodulin	
GTP-binding protein	
Receptors and membrane proteins	
Granulocyte colony-stimulating factor receptor	
G-protein-coupled receptor V28	
Interferon-γ receptor(E3H)	
Apoptosis-related proteins	
DAD-1 (defender against cell death 1)	
Bcl-2 related (Bfl-1)	
GADD153	
RNA binding protein	
Double-stranded RNA-binding protein	
Protein modulation	
Ubiquitin-related protein SUMO-1	
Ubiquitin-activating enzyme E1	
Interferon signaling related	
Interferon regulatory factor 1	
RIG-G	
Interferon-inducible 56-kDa protein	
(2'-5') Oligoadenylate synthetase	
Cell cycle regulators	
CDK inhibitor (p19INK4d)	
p21Cip1 CDK inhibitor	
Cell surface antigens and cell adhesion proteins	
CD11b (p170)	
LFA-1 CD11a	
CD11c	
Metabolism	
Dioxin-inducible cytochrome P450	
Transglutaminase	

Abbreviations: ATRA, all-*trans*-retinoic acid; CEBPε, CCAAT enhancer binding protein ε; CDK, cyclin-dependent kinase; LFA, leukocyte function-associated antigen.

repression by the POZ/BTB-CoR association. One recent clinical report showed that CR could be attained in *PLZF-RARα*-positive patients with the combined use of ATRA and G-CSF, which has also been shown to inhibit HDAC activity *(97)*. In another study, sodium phenylbutyrate, an inhibitor of HDAC, was successfully used with ATRA to achieve CR in a case of t(15;17) APL with resistance to ATRA alone *(98)*.

4.3.2. Gene Expression Profile Modulated by ATRA

Although the interaction between ATRA and the aberrant as well as wild-type RA receptor-CoR/CoA complexes has been largely elucidated, the molecular events downstream of the RA receptor complexes remains obscure. Recently, several groups including our own have addressed this issue by identifying genes modulated by ATRA *(99–103)*.

Using techniques that allow relatively large-scale transcriptional expression analysis, such as cDNA microarrays, differential display-PCR (DD-PCR), and suppression subtractive hybridization (SSH), the investigators found the expression patterns of a large number of genes to be modulated in the APL cell line NB4 upon treatment with ATRA. For example, a list of 169 genes either upregulated (n = 100) or downregulated (n = 69) by ATRA has been published (see Tables 4 and 5 for some examples) *(103)*. Gene expression was induced within 12 h of ATRA treatment in half of the upregulated genes, and over 24–72 h of treatment in the other half. By contrast, among the downregulated genes, >90% showed decreased expression levels within 8 h of treatment, and only a few with reduced expression after 12 h. A cycloheximide inhibition test indicated that the transcriptional expression of 8 up- and 24 down-regulated genes was independent of protein synthesis.

Table 5
Examples of Genes Downregulated by ATRA in the Differentiation of APL Cells

Transcription factors and general DNA binding proteins	
MYC	
MYB	
Proliferation-associated protein PAG	
DNA synthesis/repair and recombination proteins	
DNA-repair helicase (ERCC3)	
DNA-repair protein XRCC1	
DNA topoisomerase II α isozyme	
Cytokines and chemokines	
Hepatoma-derived growth factor	
Signal transduction modulators and effectors	
MAP kinase p38	
MAP kinase kinase 3 (MKK3)	
ERK3 (extracellular signal-regulated kinase 3)	
cAMP phosphodiesterase HPDE4A6	
Protein modulation	
Heat shock 60-kDa protein 1	
Cell cycle regulators	
Cyclin B1	
Cyclin A	
Apoptosis-related proteins	
ICH-1 protease short isoform	
Fast kinase	
Cell structure/mobility	
HDLC1 (cytoplasmic dynein light chain 1)	
Cell adhesion proteins	
Integrin-α 2B [platelet membrane glycoprotein IIB (GPIIb); antigen CD41B]	
Metabolism	
Superoxide dismutase 1	
Aminopeptidase N	

Functionally, these modulated genes seemed to present a picture of a well-coordinated network. The initiation of differentiation was orchestrated with the arrest of cellular proliferation, as reflected by downregulation of genes involved in cell cycle-positive control and DNA synthesis/repair. Some genes associated with apoptosis were modulated in a way that favored differentiation. Several cytosolic signal transduction pathways were implicated in ATRA-induced differentiation, such as the upregulation of cAMP/protein kinase A (PKA) and interferon/signal transducer and activator of transcription (STAT) pathways and the downregulation of the mitogen-activated protein kinase (MAPK) pathway. Of note, a recent study found that CCAAT enhancer binding protein ε (C/EBPε) could be induced in a ligand-dependent manner by PML-RARα but not by PLZF-RARα, lending strong support to the hypothesis that these two chimeric receptors have different target gene specificities *(104)*.

4.3.3. Role of the cAMP Pathway

Several lines of evidence suggest that the cAMP/protein kinase A (PKA) pathway may also play an important role in the differentiation of APL cells induced by ATRA. First, in NB4–R1 cells, there was a decoupling between ATRA "priming" and cAMP/PKA "triggering" processes *(105)*. As a result, ATRA was not sufficient to induce the maturation of these cells. On the contrary, the combination of ATRA and cAMP was able to overcome the differentiation defect. Second, increasing cAMP synthesis in the cells could reduce the concentration of ATRA required to trigger APL cell differentiation to near physiologic levels, whereas the addition of cAMP antagonist impaired the maturation of RA-sensitive APL cells *(106)*. Third, a recent report showed that in NB4–R2 cells harboring a dominant negative mutation in the E domain of PML-RARα and thus no longer able to respond to ATRA, the RXR agonist in combination with cAMP could induce terminal differentiation *(107)*. A study at the Shanghai Institute of Hematology showed that there could be a gene expression network regulated by the cAMP pathway in ATRA-induced maturation and that a specific inhibitor of PKA could at least partly block the differentiation of APL cells (J. Tao, J. W. Zhang, and Z. Chen, unpublished data).

4.4. Mechanisms Underlying As_2O_3 Treatment

4.4.1. Arsenic Trioxide Exerts Dose-Dependent Dual Effects on APL Cells

In in vitro studies, As_2O_3 shows a biphasic effect on the APL cell line NB4 and fresh APL cells *(108)*. At relatively high doses (0.5–2 μ*M*, which correspond to in vivo plasma concentration during and shortly after intravenous drug administration), As_2O_3 triggers apoptosis, as demonstrated by decreased cell viability, typical apoptotic morphology (condensed chromatin and nuclear fragmentation), increased sub-G1 cells on flow cytometric analysis of cellular DNA content, DNA ladder pattern in agarose gel, and increased expression of annexin V on the cell surface membrane. When APL cells are incubated with low concentrations of the drug (0.1–0.5 μ*M*, the plasma concentration of drug during most of the in vivo treatment period) for relatively long times (7–12 d), the cells tend to undergo differentiation *(108,109)*. Not only is there morphologic maturation, exemplified by an increased cytoplasmic/nuclear ratio, condensation of chromatin structure, and the appearance of neutrophilic granules in the cytoplasm, but also the emergence of functional markers and properties, such as CD11b expression, decreased expression of CD33, and increased cell adhesion. However, this differentiation is not terminal, compared with that induced by ATRA, since most cells are blocked at the myelocyte/metamyelocyte stage of differentiation and the nitroblue tetrazolium (NBT) reduction test is negative.

4.4.2. High-Dose As_2O_3 Induces Apoptosis via the Mitochondrial Pathway and Depends on Thiols in Target Cells

Mitochondria were recently demonstrated to be the major cellular machinery controlling apoptosis. The collapse of mitochondrial transmembrane potentials (ΔΨm) and the opening of the mitochondrial permeability transition (PT) pore allow the release of cytochrome C and other apoptosis-inducing factors (AIFs) to enter the cytoplasm. These factors, in turn, activate the caspase cascade, ultimately triggering apoptosis *(110)*. To test whether As_2O_3 affects ΔΨm and PT, investigators at the Shanghai Institute of Hematology designed experiments using double staining of cells with PI, a membrane-impermeable DNA-binding dye, and rhodamine 123 (Rh123), a cationic lipophilic fluorochrome taken up by mitochondria in proportion to the ΔΨm *(109)*. It was shown that untreated APL cells are characterized by negative staining with PI (intact plasma membrane) and high Rh123 staining (normal ΔΨm). With the As_2O_3 treatment (1.0 μ*M*) for 1–3 d, APL cells with negative PI and low Rh123 staining (disrupted ΔΨm) increased in a time-dependent manner. These changes coincided with As_2O_3(1.0 μ*M*)-induced apoptosis in the APL cells.

Meanwhile, transmission electronic microscopic examination showed that these apoptotic cells presented dense mitochondrial matrix in addition to condensed chromatin and fragmental nuclei. As expected, the activity of caspase-3 was significantly increased over 12–24 h of treatment with 1.0 μ*M* As_2O_3. Interestingly, the effects of high-dose As_2O_3 are not restricted to APL cells, because growth inhibition and/or apoptosis can also be induced in cultured malignant lymphocytes, myeloma cells, and some solid tumor cell lines, such as esophageal carcinoma and neuroblastoma *(111)*. In these cell systems, apoptosis associated with disruption of ΔΨm seems to be a common mechanism. Recently, it was shown that a c-Jun N-terminal kinase-dependent, p53-independent pathway *(112)* and a tubulin pathway *(113)* are involved in As_2O_3-induced apoptosis. Of note, in APL cells, As_2O_3 seems able to downregulate the expression of *BCL-2*, a well-known antagonist of apoptosis *(56)*.

It has been known since the begining of the 20th century that the major biologic effects of trivalent arsenic compounds are based on their binding to the adjacent sulfhydryl (-SH) groups present in biomolecules to form a five-member ring structure *(114)*. Many -SH group-containing proteins, including some enzymes, are thus targets of As_2O_3. Of note, one protein involved in the regulation of the PT pore, adenine nucleotide translocator (ANT), could be a target of As_2O_3, because its configuration is regulated through the redox status of the -SH groups of two adjacent cysteines. Cytokinin (CK), voltage-dependent anion channel (VDAC) and peripheral

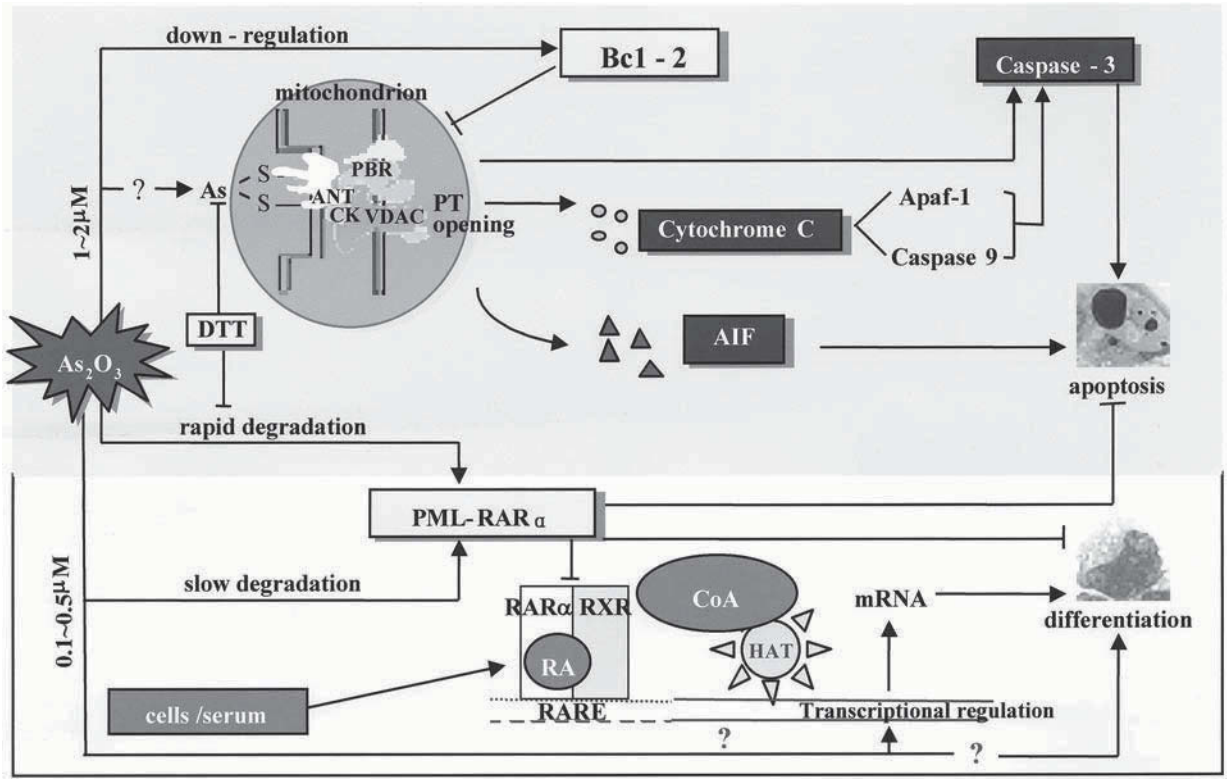

Fig. 4. Working hypothesis for the mechanism of action of As_2O_3 in remission induction of APL. As_2O_3 induces remission of APL patients via two independent pathways, i.e., by triggering partial differentiation and inducing apoptosis. High-dose As_2O_3-induced apoptosis involves mainly the -SH group-related $\Delta\Psi m$ collapse owing to the opening of the permeability transitionn (PT) pore, which leads to the release of pre-apoptotic factors from mitochondria to cytoplasma, followed by caspase activation and degradation of specific substrates. On the other hand, low-dose As_2O_3-induced differentiation might be related to retinoic acid receptor/retinoid X receptor (RAR/RXR) signaling pathway(s) and/or regulation of histone acetylation. As_2O_3 at both high and low dose triggers the degradation of PML/RARα oncoprotein. →, stimulation; —|, inhibition. AIF, apoptosis-inducing factor; ANT, adenosine nucleotide translocator; Apaf-1, apoptosis-activating factor-1; CK, cytokinin; CoA, coactivator; DTT; dithiothreitol; HAT, histone acetylase; PBR, peripheral benzidiazepine receptor; RA, retinoic acid; RARE, retinoic acid response element; VDAC, voltage-dependent anion channel.

benzidiazepine receptor (PBR) are other proteins known to be involved in the regulation of PT pore opening (Fig. 4) *(114)*. Interestingly, when the -SH groups were protected by dithiothreitol (DTT), a widely used disulfide bond-reducing agent, most of the effects by high-dose As_2O_3 could be blocked, including the As_2O_3-induced $\Delta\Psi m$ collapse, activation of caspase-3, and apoptosis. By contrast, when a selective inhibitor of γ-glutamylcysteine synthetase, buthionine sulfoximine (BSO), was used, APL cells became more sensitive to As_2O_3. In agreement with these results, it has been shown that the cellular GSH content and the activity of some antioxidant enzymes, such as catalase, are determinants of drug sensitivity *(109)*.

4.4.3. Low-Dose As_2O_3-Induced Cell Differentiation and the RA Pathway

Relatively little is known about the mechanisms by which As_2O_3 induces APL cell differentiation. In contrast to the effects of high-dose arsenic, differentiation induced by low concentrations of As_2O_3 is not blocked by DTT, suggesting that other pathways are involved. Of note, the ATRA-resistant NB4-derived sublines MR2, R1, and R2 were sensitive to the apoptosis-inducing effects of high-dose As_2O_3, but they did not respond to treatment with As_2O_3 at 0.1–0.5 μ*M*. Moreover, the ATRA-sensitive HL60 cell line, but not the ATRA-resistant $HL60^{Res}$ clone, could also be induced to differentiate along the granulocytic pathway, as confirmed by elevated CD11b and CD14 expression, decreased CD33 expression, and NBT reduction.

It is worth pointing out that NB4-R2 cells bear a mutated *PML-RARα* whereas $HL60^{Res}$ cells harbor a mutation of *RARα*. As previously mentioned, relapsed APL patients who lose *PML-RARα* expression are generally insensitive to remission induction with As_2O_3. When the gene expression profiles in APL cells exposed to low-dose As_2O_3 were compared with those under physiologic concentrations of ATRA, some genes investigated showed similar patterns of expression modulation by these two drugs. All these observations suggested the possibility that the mechanisms of low-dose As_2O_3-induced differentiation might be associated with an RA regulatory pathway *(109)*. However, our recent study found that RARα-specific antagonist did not block the differentiation-inducing effect of As_2O_3, whereas the drug showed

very weak effect on the expression of a reporter gene driven by RARE (Y. L. Shen, G. Q. Chen, and Z.Chen, unpublished data). Therefore, low-dose As_2O_3 seems not to act directly on the transactivation properties of RARα. It will be interesting to investigate, in the future, whether As_2O_3 exerts any influence on cellular transcription regulatory mechanisms such as the deacetylation/acetylation of histones.

4.4.4. As_2O_3 Targets the PML-RARα Fusion Protein

The most striking finding in exploring the molecular mechanisms of arsenic treatment in APL has been the As_2O_3-induced modulation/degradation of the PML-RARα oncoprotein *(108,115,116)*. Under the effect of 1 μ*M* As_2O_3, the PML staining patterns changed drastically in APL cells. Within 12 h, the micropunctates were targeted into the PODs, which further aggregated at 24 h. PML staining had almost disappeared by 48 h. Western blot analysis with the anti-RARα antibody showed degradation of PML-RARα, but not RARα, within 24 h of treatment. Similar changes occurred under low concentrations (0.1 μ*M*) of As_2O_3, although the kinetics were slower. In addition, in non-APL cells, As_2O_3 could also modulate the distribution of wild-type PML protein and could cause its degradation. PML was recruited from nucleoplasm to PODs and formed high-molecular-weight conjugates with SUMO-1 before degradation *(116)*. Since *PML-RARα* plays a central role in APL pathogenesis, including arrest of differentiation and deregulation of apoptosis, its degradation induced by As_2O_3 should favor the restoration of the differentiation/apoptosis programs. As a result, the wild-type RAR/RXR pathway may be opened up and the partial differentiation of APL cells induced by the physiologic concentrations of RA. Figure 4 illustrates our working hypothesis for the mechanism of As_2O_3 action in remission induction therapy for APL.

5. PERSPECTIVES

So far, selective cell differentiation therapy has been successful only in APL. Thus, what can we learn from the APL model? Can the concept of differentiation therapy be extended to other leukemias and solid tumors to benefit more patients?

We believe the major lesson to be gotten from ATRA/As_2O_3 treatment of APL is that both drugs target the oncoprotein PML-RARα *(4,64)*. With rapid developments in the human genome project and cancer research in general, it is possible that all major genetic abnormalities underlying the human leukemias and solid tumors will be identified before the end of the first decade of the 21st century. This achievement will undoubtedly inspire the development of therapies targeted to the specific genetic defects that characterize the different human cancers. The "targeting" approach, together with more rational use of relatively nonspecific chemotherapy, may significantly improve the outcome of cancer treatment. Of course, the mechanisms underlying treatment with arsenic compounds may be more complex than those for ATRA and should be further explored.

Another inspiration that we may draw from the APL model is that basic research on protein-protein and protein-DNA interactions in some common pathways involved in cancers may lead to important new targets for therapeutic agents *(64)*. PML-RARα is associated with CoR/CoA and thus linked to one of the key elements in transcriptional regulation, the control of histone deacetylation/acetylation. Hence, ATRA differentiation therapy can be considered, after all, a therapy based on reprogramming of transcriptional repression/activation. As a matter of fact, the fusion gene products in some other forms of malignant hemopathies have also been found to promote abnormal interactions of CoR/CoA. These include AML1-ETO (or MTG-8) in AML-M2b with t(8;21)(q22;q22) *(117)*, TEL-AML1 in ALL with t(12;21)(p13;q22) *(118)*, and LAZ3/BCL6 (an oncoprotein homologous to PLZF) in diffuse large cell lymphoma *(119)*. ETO, TEL, and LAZ3/BCL6 have all been found to possess the CoR binding motif. In some instances, even the genes encoding proteins with HAT activities [fusions between MOZ on 8p11 and distinct partners in AML-M4 and -M5, MLL-CBP in t(11;16)(q23;p13.3), or MLL-P300 in t(11;22)(q23;q13)] were directly involved in chromosomal translocations *(120–122)*. Recently, several groups have shown that combined use of HDAC inhibitors and G-CSF could induce differentiation of t(8;21) leukemia cells in vitro *(123,124)*. It is hoped that the research on inhibitors of HDAC (and perhaps activators of HAT as well) may open new avenues for the treatment of human malignancies.

A third major issue is the combination of differentiation/apoptosis inducers and other drugs. ATRA in combination with cytotoxic chemotherapy has been confirmed to give better clinical results than either ATRA or chemotherapy alone. An important question to be answered in the future is whether or not incorporation of As_2O_3 into the postremission treatment could yield higher 5-yr DFS rates than the ATRA/chemotherapy combination by itself. A potentially useful strategy might be to combine differentiation inducers, such as HDAC inhibitors or G-CSF, with ATRA, as predicted by the PLZF-RARα model. It is possible that these combinations targeting proteins at different levels of the same pathway may induce responses in leukemias other than APL and in solid tumors as well. As previously mentioned, ATRA in association with cAMP, or an RXR-specific agonist combined with cAMP, could overcome the differentiation arrest of APL subclones, which cannot be corrected with ATRA alone. On the other hand, 13-*cis* RA with interferon-α could produce high response rates for patients with squamous cell carcinomas of the head, neck, and cervix *(125)*. Thus, combinations of differentiation inducers for distinct but crosslinked pathways may also be tried in cancer treatment.

In conclusion, differentiation/apoptosis therapy in APL may represent one of the most promising approaches to cancer treatment in the new century, continued intensive research efforts are worthwhile.

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22 Acute Promyelocytic Leukemia

Perspective 2

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CONTENTS

1. INTRODUCTION

Acute promyelocytic leukemia (APL) is a peculiar form of acute myeloid leukemia (AML) with distinctive biologic and clinical features. These include the frequent association at diagnosis of a life-threatening hemorrhagic diathesis, the presence in leukemic blasts of a specific chromosomal translocation, t(15;17), and a unique response to differentiating therapy with retinoids *(1–7)*. Knowledge of these characteristics for prompt specific recognition and treatment has contributed in recent years to a dramatic improvement in the clinical outcome of patients affected by the disease. In most cases, APL presents as a hyperacute leukemia that can produce high rates of early death (10–15% of cases), mainly due to intractable bleeding, even in patients receiving early treatment and intensive supportive care *(8–20)*. The disease may be simultaneously defined as the most rapidly fatal human leukemia, if left untreated, and as the most frequently curable leukemia of adults, if promptly diagnosed and adequately treated. For this reason, patients need to be referred to highly specialized centers for diagnostic confirmation and for the institution of specific therapy. The latter differs considerably from therapy used for other AML subtypes. According to most recent studies, up to 70% of newly diagnosed APL patients may be cured with state-of-the-art approaches *(8–20)*. The two factors most responsible for this progress are the introduction of retinoid compounds such as all-*trans* retinoic acid (ATRA) into frontline treatment and the cloning of t(15;17), followed by development of tests for rapid genetic diagnosis and disease monitoring *(1–7)*.

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2. CLINICAL RELEVANCE OF GENETIC DIAGNOSIS

Although the morphologic characteristics of affected blast cells usually allow specific recognition of APL, confirmation of diagnosis at the genetic level is strongly recommended, not only in suspected cases, but also in morphologically typical cases. In fact, detection of t(15;17) and/or the underlying *promyelocytic leukemia-retinoic acid receptor-α* (*PML-RARα*) rearrangement is predictive of response to ATRA in virtually 100% of cases *(21–23)*. The observation of resistance in newly diagnosed patients receiving adequate ATRA treatment should raise serious concerns as to the accuracy of the initial characterization and lead to reassessment of the genetic diagnosis. ATRA has been shown to target the disease-specific PML-RARα protein, which is believed to be a mediator of ligand activity in leukemic blasts *(24–26)*. Furthermore, variant karyotypes such as the infrequent t(11;17) involving the *promyelocytic leukemia zinc finger* (*PLZF*) gene instead of PML, have similar morphologic appearances but have been shown to be unresponsive to ATRA *(27,28)*. Finally, cases with variant morphologic features resembling other AML subtypes and bearing the *PML-RARα* hybrid gene have been reported *(29)*.

It is important to remember that although detection of t(15;17) by conventional cytogenetics permits specific diagnosis, absence of the translocation does not rule out the occur-

rence of APL. In fact, some cases with an apparently normal karyotype have been reclassified as *PML-RARα*-positive by fluorescence *in situ* hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR) analysis *(30–34)*. These t(15;17) false negatives may result from technical problems with karyotyping (e.g., few metaphases, poor quality of mitoses) or to the occurrence of cryptic translocations *(30–34)*. Only anecdotal information is available at present on the clinical characteristics and response to ATRA of the rarest APL variants, which involve the *RARα* locus and partner genes other than *PML* or *PLZF*, such as *NPM*, *NuMA-RARα* , and *STAT5b (35–37)*.

For the purpose of prompt administration of specific ATRA-containing therapy, RT-PCR provides more rapid diagnosis than does FISH analysis; however, the latter is equally specific *(22,23)*. Similarly, antibodies directed to the amino-terminal part of PML, which is conserved in all types of recombinations with RARα, have proved extremely valuable for the recognition of protein disruptions due to t(15;17) *(33,34,38,39)*. With the use of either immunocytochemical or immunofluorescent assays, these antibodies allow rapid identification of the so-called microspeckled staining of the PML protein, as opposed to the speckled distribution of wild-type PML in non-APL leukemias *(33,34,38,39)*. Importantly, either RT-PCR with *PML* and *RARα* primers or PML antibody diagnostic approaches would give negative results in the ATRA-unresponsive t(11;17)-positive cases.

In clinical practice, some diagnostic issues might be controversial. For example, when should APL be suspected at the morphologic level? In which AML cases should the *PML-RARα* lesion be suspected and which methodologies should be used to confirm its presence? Finally, should ATRA be started in patients with morphologically typical APL before a genetic diagnosis is made? Answers to these questions must take into account the cost/benefit ratio. In light of the difficulty of establishing objective morphologic criteria for suspected APL, and considering the low cost of immunostaining, a suitable approach would be to include the anti-PML antibody routinely in the diagnostic characterization panel for all AMLs. This would allow recognition of those APL cases with atypical morphology in which cytogenetic characterization is either not available or results in a normal karyotype.

Even though such cases may occur at a very low frequency, their correct diagnosis is critical in light of their high curability with specific therapy. As to the initiation of specific therapy with ATRA, it is important to emphasize that this agent has proved effective in the control of the APL-associated hemorrhagic syndrome; indeed, one of the first signs of an ATRA response in APL is the improvement of laboratory signs of the coagulopathy *(40–44)*. Thus, prompt institution of ATRA is clinically recommended. Also, in light of the fact that simultaneous use of ATRA and cytotoxic chemotherapy is nowadays considered standard care for the disease *(7,19)*, it is advisable that treatment be started immediately (at least in morphologically typical APL cases) without waiting for the results of genetic characterization. Patients who might occasionally be found not to have *PML-RARα*-positive APL upon successive genetic analyses would not be harmed by several days of ATRA therapy and then could be promptly switched to AML-like regimens.

3. FRONTLINE THERAPY

Anthracyclines and retinoids are the mainstays of therapy for APL. The observation of an exquisite sensitivity of APL to daunorubicin, originally reported by Bernard et al. *(45)* in 1973, was confirmed by other European groups in the 1980s and extended to other anthracyclines *(46–48)*. Importantly, unlike experience with other AML subtypes, these agents were able to produce long-lasting remissions when used as induction monochemotherapy for APL. Although the reasons underlying such high sensitivity are unclear, a possible important factor influencing the favorable response to anthracyclines might be the absence of the multidrug resistance glycoprotein P170 on APL cells *(49)*.

A dramatic response to treatment with oral ATRA in APL patients was initially reported by the Shanghai team in 1988 *(50)* and subsequently confirmed by other groups *(51–52)*. Complete remission (CR) rates of approx 80% were obtained with this agent in either newly diagnosed or relapsed patients by the induction of cell differentiation with no marrow aplasia *(50–52)*. These observations provided the first example of a disease-targeted biologic treatment in leukemia and fostered basic and clinical investigation on differentiation therapy. Despite the impressive initial results, it soon became clear that such remissions were short lived in patients who remained on ATRA monotherapy, prompting the inclusion of conventional chemotherapy together with ATRA for combinatorial treatment. In the 1990s, a number of large single or multiinstitutional trials of APL were conducted in the United States, Europe, Japan, and China *(7–20)*. Early studies established the advantage of including ATRA in frontline therapy and its superiority over standard chemotherapy *(8)*. In the following years, several questions were addressed in prospective studies, including the optimal ATRA plus chemotherapy schedule (sequential vs simultaneous administration), the role of maintenance therapy, and that of molecular monitoring *(7–20)*. The main results of these trials, which altogether included some 2000 patients, were reported in 1997–1999 and are summarized below (Table 1).

3.1. Summary of ATRA Clinical Trials

3.1.1. Improved CR and DFS Rates

Improved CR rates (72–95%) and 3–4-yr disease-free survival (DFS) rates (62–75%) were reported in all series, including those of the Shanghai Cooperative Study Group *(11)*, U.S. Intergroup *(14)*, Memorial Sloan-Kettering Cancer Center (MSKCC) *(15)*, MD Anderson Cancer Center (MDACC) *(16)*, European APL'93 *(19)*,Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) *(13)*, Japanese Adult Leukemia Study Group (JALSG) *(17)*, British Medical Research Council (MRC) *(18)*, and Spanish PETHEMA *(20)*, with better results obtained in patients receiving the simultaneous combination. The superiority in terms of efficacy of the upfront (ATRA plus chemotherapy) over the sequential (ATRA followed by chemotherapy) schedule was demonstrated in a randomized comparison by the European APL'93 study *(19)*. Besides resulting in better DFS rates, the simultaneous

Table 1
Main Results of Recent ATRA Plus Chemotherapy Trials in Newly Diagnosed APL

Group	*Year*	*Randomized*	*No. of patients*	*Genetic diagnosis (% pts.)*	*CR rate (%)*	*Early death rate (%)*	*Outcome*	*Ref. No.*
Chinese	1992	No	400	NA	75	NA	NA	*11*
Cooperative Group	1995	No	423[a]	NA	NA	NA	5-yr OS, 18–71%	*11*
MSKCC	1997	No	73	89	81	13	5-yr DFS, 67%	*15*
U.S. Intergroup	1997	Yes[b]	346	64	69–72	11–14	3-yr DFS, 32–67%	*14*
MDACC	1997	No	43	95	77	19	1.5-yr DFS, 80%	*16*
JALSG	1997	No	196	NA	88	9	4-yr DFS, 62%	*17*
GIMEMA	1998	No	480	100	95	7	4-yr DFS, 75%	*55*
MRC	1999	Yes[c]	239	91	70–87	12–23	4-yr OS, 52–71%	*18*
APL'93	1999	Yes[d]	413	93	90–95	7	2-yr EFS,77–84%	*19*
PETHEMA	1999	No	123	100	89	10	2-yr DFS, 92%	*20*

Abbreviations: NA, not available; CR, complete remission; OS, overall survival; DFS, disease-free susrvival; EFS, event-free survival; ATRA, all-*trans*-retinoic acid. Definitions of other abbreviations may be found in the text.
[a]ATRA vs chemo vs. ATRA + chemo (two protocols) as postremission therapy.
[b]ATRA vs chemo.
[c]Short (5-d) course of ATRA prior to chemo vs extended ATRA begun with chemo.
[d]ATRA given simultaneously with vs after chemo.

approach appeared more effective in diminishing the occurrence of overt ATRA syndrome *(11–20)*.

3.1.2. Maintenance Therapy

Both the European APL'93 *(19)* and the U.S. Intergroup *(14)* trials showed the advantage of including ATRA-containing maintenance in the treatment program. In addition, results of the European APL'93 study *(19)* suggested further benefit from maintenance with combined ATRA plus low-dose chemotherapy with methothrexate and 6-mercaptopurine. This issue is also being investigated by the GIMEMA group, which adopted the same four randomization arms of the APL'93 study (ATRA vs chemotherapy vs. ATRA + chemotherapy vs observation) *(13)*. Updated results of the GIMEMA study also confirm a better outcome for patients receiving ATRA (unpublished data).

3.1.3. Prospective RT-PCR Analyses

Results of the MSKCC *(15)*, MRC *(18)*, GIMEMA *(13)*, and PETHEMA *(20)* studies highlighted the value of prospective RT-PCR analyses of *PML-RARα* for refined diagnosis and better assessment of response to therapy.

3.1.4. Decrease in Morbidity and Mortality

The morbidity of the life-threatening ATRA syndrome was successfully counteracted by strict adherence to the recommendations of the MSKCC group, which include rapid institution of high-dose dexamethasone at the earliest manifestations of the syndrome (fever, respiratory distress, pulmonary infiltrates, pleural effusions, and weight gain) *(53)*. Following these rules, death rates attributable to the ATRA syndrome have now dropped to 1–3% in patients receiving the simultaneous ATRA plus chemotherapy schedule *(13–20)*.

Together, these data show a dramatic improvement in the outlook for patients with APL, up to 70% of whom are being reported as long-term survivors, compared with historical figures of 30–40% obtained in the pre-ATRA era. In spite of this progress, some 30% of patients receiving state-of-art therapy still die of their disease due to early death or, more frequently, relapse *(13–20)*.

3.2. Remaining Controversies

Even though agreement has been reached on some issues, including the importance of genetic diagnosis, therapy for ATRA syndrome, and the benefit of the ATRA/chemotherapy upfront schedule and of ATRA maintenance, divergent opinions remain on other issues that will probably be the subject of further investigation in the years ahead. For example, the measures to prevent and treat the hemorrhagic diathesis in APL patients has not been standardized and are usually left to the physician's discretion in multiinstitutional studies. In this respect, most relevant controversies concern the use of heparin of antifibrinolytic agents as well as policies for platelet and plasma support, particularly as regards their prophylactic use. Finally, although the approach to managing overt ATRA syndrome has been clearly established by the MSKCC team *(53)*, the effectiveness of preventive measures, such as low-dose corticosteroid administration, is still unclear. However, a reduction in pulmonary toxicity was demonstrated in a small multicenter study by the Australian Leukemia Study Group (ALSG) with use of prednisolone prophylaxis (75 mg/d) during ATRA treatment *(54)*. Other investigational areas for clinical research include the: (1) type and intensity of induction and consolidation chemotherapy; (2) role of cytarabine, and (3) place of novel agents such as other retinoids and arsenic trioxide.

The type and intensity of induction and consolidation chemotherapy have varied considerably in reported trials. In fact, AML-like regimens including cytarabine have been used in most of the studies noted above [U.S. Intergroup *(14)*, European APL'93 *(19)*, MRC *(18)*, JALSG *(17)*], whereas tailored anthracycline-based chemotherapy has been employed in addition to ATRA for remission induction by the GIMEMA *(13)*, MDACC *(16)*, and PETHEMA *(20)* groups. Furthermore, the latter two groups also omitted non-intercalating agents from

the consolidation phase, with no apparent reduction of therapeutic efficacy *(16,20)*. The PETHEMA study *(20)* showed a substantial benefit of an anthracycline-based consolidation phase, with absence of deaths in remission and an overall reduction in toxicity. Two other studies further support the notion that cytarabine might have a minor role in the treatment of APL: (1) a randomized GIMEMA trial conducted in the pre-ATRA era, which compared cyatarabine plus idarubicin vs idarubicin during induction and showed similar results in the two arms *(55)*; and (2) a recent metaanalysis of patients entered in the GIMEMA and PETHEMA ATRA trials. The latter study was performed with the aim of establishing whether the addition of cytarabine and other non-intercalating agents during consolidation, as done in the GIMEMA trial, resulted in better relapse-free survival *(56)*. Patients in both series received identical induction and maintenance regimens, and treatment differed in whether or not cytarabine and other non-intercalating agents were included during consolidation. No significant differences were found in relapse-free survival, suggesting that omission of cytarabine might allow better adaptation to the ATRA/anthracycline combination according to risk category *(56)*.

3.3. Role of Arsenic Trioxide and Other Agents

Very promising results with arsenic trioxide were initially reported from China for relapsed APL *(57)* and were confirmed by the MSKCC group *(58)*. These investigators showed that arsenic trioxide selectively induces partial differentiation and apoptosis in APL and that remission is achieved in most patients at the molecular level after two or three cycles of treatment *(58)*. Most importantly, this agent appeared effective in patients relapsing after ATRA treatment and was shown to overcome resistance in vivo in APL cells bearing mutations in the *RARα* ligand binding domain *(57–59)*. This latter feature is believed to represent a major mechanism accounting for the development of ATRA resistance; thus, the observation of remission induction with arsenic trioxide in these patients provided evidence of a non-cross-resistant action of this agent with respect to ATRA. Finally, although synergistic action between arsenic trioxide and ATRA has not yet been documented in vivo, it has been demonstrated in vitro as well as in animal models *(60,61)*, opening an interesting perspective for combination treatment with these two agents. Some investigators have proposed the inclusion of this agent in frontline therapy, and ongoing trials in Europe and the United States are testing the role of arsenic trioxide for high-risk APL (e.g., patients with elevated initial white blood cell count) or during consolidation. Although the results of these trials should provide relevant information, some concerns over the use of frontline arsenic trioxide were raised by a recent Chinese study in which lethal hepatic toxicity developed in 2 of 11 newly diagnosed patients *(62)*.

Other still unsolved issues have to be considered before this promising agent can be included in frontline therapy for newly diagnosed APL, i.e., long-term therapeutic efficacy and toxicity. Preliminary data suggest that molecular remission is short lived in patients who receive arsenic trioxide alone, thus indicating the need to consolidate these remissions with more cytotoxic therapy *(62)*. Taken together, presently available information indicates that arsenic trioxide has an important role in the treatment of APL; however, further investigation is warranted to establish its optimal place better in the management of this disease.

With respect to other retinoids, the naturally occurring 9-*cis* retinoic acid (9-*cis* RA) and the synthetic derivative Am80 have been shown to be effective in relapsed patients *(63–65)*. Compared with ATRA, 9-*cis* RA shows broader activity, as it binds to both the RAR and RXR classes of receptors *(63)*. In addition, in vitro studies showed differential binding of 9-*cis* RA to distinct PML-RARα isoforms, with higher affinity for the bcr3 (short) type *(66)*. Am80 has proved to be approx 10 times more potent than ATRA in vitro, being required at much lower concentrations to exert an equivalent effect on cell differentiation *(64,65)*. Because none of these agents have been tested in vivo in significant numbers of patients with advanced disease, it is still unclear whether their activity is superior to that of ATRA.

Investigators at the MDACC in Houston recently tested liposomal encapsulated ATRA as monotherapy for newly diagnosed APL. In a preliminary analysis, these authors reported prolonged molecular remission (i.e., PCR negativity for *PML-RARα*) in most evaluable patients *(67)*. In spite of the low patient number and limited follow-up, this study strongly suggests that the liposomal ATRA formulation is more effective than orally administered ATRA and that the amount of chemotherapy needed to cure the disease might be reduced if it were used in combination with liposomal ATRA. In this respect, an interesting investigational area might be the use of liposomal ATRA in conjunction with conventional chemotherapy for high-risk patients.

In summary, the existing body of clinical information indicates that frontline therapy of APL should include for induction a simultaneous combination of ATRA and chemotherapy. The latter should contain an anthracycline, which may also be used as a single agent in conjunction with ATRA. As for consolidation, two cycles with an anthracycline backbone are usually recommended and might suffice; however, several combinatorial treatments, such as ATRA and/or arsenic trioxide together with chemotherapy, are being investigated. Finally, patients should receive an ATRA-containing maintenance therapy whose optimal schedule and duration remain to be defined. Among promising investigational areas, the possibility of adapting treatment intensification to patient risk is of utmost importance, particularly with regard to early death and the risk of disease relapse. In this respect, recent analyses of prognostic factors strongly suggest that patients with a high leukocyte count at diagnosis may require modified therapy (see Section 4).

4. PROGNOSTIC FACTORS

In patients with genetically proven APL who receive standard therapy including ATRA and anthracycline-containing chemotherapy, the achievement of hematologic complete remission (HCR) is negatively influenced only by early death, mainly due to severe hemorrhage *(1–7)*. Adverse risk factors for HCR, which by extension influence the event-free survival (EFS), are older age and elevated white blood cell count *(17,18)* (Table 2). Other features affecting the early death rate are the presence of purpura and low platelet numbers at diagnosis *(17)*. As to remission duration and relapse risk, a white blood cell

count >10 × 10^9/L at presentation was the only factor that correlated with increased relapse risk in all reported series *(17,18, 56, 68)*, retaining its significance in multivariate models in the JALSG, MRC, GIMEMA, and PETHEMA studies *(17,18,56)*. Apart from confirming the prognostic value of this feature, both the European APL'91 group *(68)* and the GIMEMA and PETHEMA groups *(56)* reported that an initial platelet count < 50 × 10^9/L or 40 × 10^9/L, respectively, also affected the relapse risk negatively. In particular, a platelet count < 40 × 10^9/L was an independent variable associated with increased relapse risk in the combined GIMEMA and PETHEMA study *(56)*, leading these investigators to construct a relapse risk model, based on initial platelet and white blood cell (WBC) count, for APL patients receiving AIDA-like regimens. The model segregated patients into low (WBC < 10 × 10^9/L and platelet >40 × 10^9/L), intermediate (WBC < 10 × 10^9/L and platelet < 40 × 10^9/L), and high-risk (WBC > 10 × 10^9/L) groups, each with a distinctive relapse-free survival curve ($p < 0.0001$) *(56)*. This finding provides a rationale for the design of risk-adapted therapies, which are being developed and tested by the PETHEMA and GIMEMA groups.

In addition to clinical features, a number of biologic characteristics have been analyzed for their impact on prognosis, including immunophenotypic markers *(69–71)*, additional karyotypic lesions *(72–75)*, type of *PML-RARα* isoform *(13,18,76–80)*, and PCR status for *PML-RARα* during follow-up *(21–23,81–89)*. Although the prognostic significance of CD2 and CD34 remains controversial *(69)*, two recent studies indicated that CD56 would be associated with inferior outcome *(70,71)*. In one such study, conducted in patients treated with upfront ATRA and idarubicin, CD56 expression was shown to be an independent marker of poorer survival in a multivariate analysis *(71)*. However, because inclusion of this antigen in the diagnostic characterization panel of the acute leukemias is relatively recent, only a minority of APL patients were characterized for CD56 expression. Thus, further studies are needed to clarify the prognostic value of this marker. As for secondary cytogenetic abnormalities, these do not seem to confer an inferior outcome in most reported series *(72–75)*, whereas a single study performed in the United Kingdom suggested that complex abnormalities were indeed associated with poorer outcome *(73)*.

A trend toward an inferior outcome was also suggested for patients with the short (or bcr3) PML-RARα isoform compared with cases with the more frequent long type (or bcr1-2) *(18,56)*. However, except for an early study in patients treated with ATRA alone *(76)*, the association of the bcr3 isoform with a poorer outcome was not statistically significant in recent ATRA plus chemotherapy trials *(18,19,56,77,78)*. Unfortunately, the majority of U.S. and European studies used methodologies that fail to distinguish the bcr1 from bcr2 PML-RARα isoforms (the latter is also referred to as "variant" form) *(18,19,56,77,78)*. Thus, the impact of including bcr2 patients in the long transcript group is unknown; however, bcr2-positive patients account for only 8% of APL cases *(1–7)*. A decreased sensitivity to ATRA in vitro was initially reported for bcr2-positivepatients *(79)*, whereas a subsequent in vivo study in patients enrolled in the U.S. Intergroup trial did not entirely clarify this issue, most likely because of the low number of patients studied and the fact that half of them received suboptimal therapy (chemotherapy alone) *(80)*. Finally, the slow kinetics of molecular remission and persistence of or conversion to PCR positivity for *PML-RARα* after consolidation have been correlated with increased risk of hematologic relapse *(18)*.

Table 2
Prognostic Factors in APL Patients Receiving ATRA Plus Chemotherapy

Factors	*CR*	*DFS*	*Ref. No.*
Clinical			
Old-age	Yes	Yes	*17–20*
Low WBC count	Yes	Yes	*17–20*, *56*
Purpura	Yes	No	*17*
High platelet count	Yes	Yes	*56*, *68*
Biologic			
Additional chromosomal lesions	No	No	*75*
PML-RARα isoform	No	No	*13*, *18*, *56*
CD56	No	Yes	*70*, *71*
Slow loss of PCR positivity	—	Yes	*18*

Abbreviations: ATRA, all-*trans*-retinoic acid; CR, complete remission; DFS, disease-free survival; WBC, white blood cell; *PML-RARα*, promyelocytic leukemia/retinoic acid receptora; PCR, polymerase chain reaction.

5. THE ROLE OF MINIMAL RESIDUAL DISEASE MONITORING

Apart from its value in diagnostic refinement, the RT-PCR technique offers a powerful tool for sensitive assessment of response to treatment *(21–23)*. Following initial retrospective studies *(81–84)*, which suggested the clinical utility of using PCR monitoring in APL, several groups prospectively evaluated the significance of sequential PCR analysis in patients receiving uniform therapies *(13,18,20,89)*.

Using PCR tests with sensitivity levels of 1 × 10^{-4}, GIMEMA, MRC, and PETHEMA investigators found that approximately 50% of patients in hematologic remission after induction had detectable *PML-RARα* transcripts in their marrow cells *(13,18,20)*. No correlations were found between PCR status at the time of remission achievement after induction and relapse risk. After completion of consolidation, 90–95% of patients tested PCR-negative in the MSKCC, MRC, GIMEMA, and PETHEMA series *(13,18,20)*. In the MRC analysis, detection of residual disease at the end of the third chemotherapy course (of four total) predicted an increased risk of relapse *(18)*. Posttreatment monitoring was prospectively performed at preestablished times in patients enrolled in the GIMEMA study. The results indicated that conversion from PCR negativity to PCR positivity after consolidation therapy was uniformly associated with subsequent hematologic relapse, leading the Italian study group to anticipate salvage therapy in these patients *(89,90)*.

Although there is general agreement on the value of PCR positivity during remission as a predictor of relapse *(21–23)*, several cautionary issues have to be considered before therapeutic decisions are based on molecular tests. The above results are in fact based on prospective studies performed in the context of uniform clinical trials and employing PCR tests with 1×10^{-4} sensitivity. Persistence of residual disease during long-term remission, detected with more sensitive assays, has been occasionally reported *(91)*. Moereover, to rule out contamination, it is important to confirm molecular relapse in an additional marrow sample before initiating salvage therapy *(90)*. Finally, one major limitation of the assays now in use is their failure to quantitate the amount of residual disease precisely, which in turn makes comparisons among reported studies quite difficult *(22,23)*. The newly developed real-time PCR technology holds considerable promise of providing adequate quantitative standardization and therefore may promote more objective analyses of residual APL.

6. SALVAGE THERAPY

Approximately 20–30% of patients receiving upfront ATRA plus chemotherapy will eventually relapse *(11–20)*. Owing to the low numbers and heterogeneous treatment approaches in the few reported series, the optimal therapy for APL relapse is not defined *(1–7)*. Despite the high probability of achieving a second remission with ATRA or arsenic trioxide, relapsed patients are at greatest risk of early death and require intensive postremission therapy, possibly including autologous or allogeneic bone marrow transplantation. The following factors may be relevant for risk assessment and therapeutic decision making: (1) age and leukocyte count at presentation; (2) type of frontline treatment; (3) duration of first hematologic or molecular CR; (4) time elapsed since last ATRA administration; and (5) PCR status after reinduction therapy. A second response to ATRA is usually observed in patients who received this agent as part of their frontline treatment, provided that at least 6 mo have elapsed since the last administration *(6,7)*. However, its use with simultaneous chemotherapy appears more appropriate, especially in light of the better results being obtained with this schedule in frontline therapy.

Arsenic trioxide appears to be a highly effective treatment for patients relapsing after ATRA treatment *(57–59)*; however, both ATRA- and arsenic-induced remissions require intensive chemotherapy consolidation, usually followed in most institutions by allogeneic BMT for eligible patients with an HLA-identical donor. Alternatives for noneligible patients include the use of autologous stem cell infusion. The PCR status after reinduction and consolidation appears to be extremely important for further therapeutic decisions. Patients remaining PCR-positive at this stage should not receive autologous transplants, as they have been reported to relapse invariably during follow-up *(92)*. Unfortunately, the only comprehensive survey of allogeneic and autologous BMT in APL published to date includes patients treated in the pre-ATRA era *(93)*.

A recent study by the GIMEMA group suggested that anticipation of salvage therapy at the time of molecular relapse resulted in prolonged survival compared with treatment results for patients in overt relapse *(90)*. Although this approach is undoubtedly beneficial in terms of reduced early mortality, longer follow-up of the GIMEMA study and the analysis of larger series is needed to verify whether such strategies secure longer remission durations.

As stated above, only scant information is available on APL patients relapsing at the hematologic or molecular level in the post-ATRA era. Thus, in view of the small number of patients available for study, it appears that cooperative studies at the multinational level will be needed to assess the possible treatment options.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Early death and disease relapse are the main obstacles to permanent cure in APL *(7)*. These two events and their underlying causes therefore represent the most important topics for clinical research in the years ahead. With regard to the first problem, a special effort should be made to identify better the risk factors associated with severe hemorrhage at presentation and to establish measures to prevent and counteract the hemorrhagic syndrome more effectively. In the meantime, a better understanding of the pathophysiology of APL-associated coagulopathy is needed, as it may provide important clues for innovative therapeutic approaches in this specific setting.

Identification of risk categories at diagnosis for more appropriate treatment stratification remains a major challenge for future clinical investigation. The first results of risk-adapted protocols based on presently defined risk categories will be available soon and should clarify which therapeutic options are likely to improve the outcome of patients with elevated WBC counts and whether other classification systems are needed for more effective treatment modification. Studies in which the treatment approach has been simplified by decreasing chemotherapy intensity have already suggested that a fraction of APL patients are currently being overtreated and that they might therefore be spared the risk of treatment-related toxicity by judicious reductions in therapy *(20)*. In this setting, molecular monitoring might represent a useful tool for effective clinical management, in which a gradual attempt is made to decrease the intensity and length of treatment *(22,23)*. Finally, the long-term advantage of anticipating salvage treatment at the time of molecular recurrence remains to be clarified.

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CHEMOTHERAPEUTIC STRATEGIES

II

CHILDHOOD LEUKEMIAS IN UNDERPRIVILEGED COUNTRIES

K

23 Treatment of Childhood Leukemias in Underprivileged Countries

BHARAT AGARWAL AND RASHMI DALVI

CONTENTS

1. INTRODUCTION

Childhood acute lymphoblastic leukemia (ALL) is a remarkable success story in pediatric oncology. At present in the developed world, two-thirds or more of children with ALL can be cured with contemporary treatment *(1)*. Efforts are now being made to enhance the efficacy of the existing regimens while reducing their toxic side effects based on advances in the molecular and immunologic characterization of leukemic cells *(2)*. These progressive developments in the management of leukemias in children have unfortunately not translated into reality in the "developing world." Huge inequities exist in the management of children with leukemias in high- and low-income countries. It is estimated that only 1 of 10 children with acute leukemia receive any kind of treatment in these less privileged countries *(3)*. Hence, even though 70% of childhood ALL is currently curable, it is not 70% curable *worldwide*, since 90% of the world's children do not have access to curative treatment *(4)*. The success rates among the children who receive treatment are also far from satisfactory. As one could easily fathom, two major issues are involved in the management of children with leukemias in the developing countries: the first is to ensure access to health care *for all the children* with leukemia, and the second is to improve the survival rates among those who do receive treatment. There are numerous problems facing both the patients and their families, as well as the physicians involved in the care of children with leukemia. The aim of this review is to highlight these problems, which are well known, and to suggest means and methods to circumvent them, so as to achieve the goal of improved survival for ALL-affected children from underprivileged countries.

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2. LEUKEMIA: WHAT IS THE BURDEN?

Each year, more than 12 million children under the age of 5 yr die from the effects of disease and inadequate nutrition, which translates to 33,000 children per day or 1400 per hour *(5)*. The vast majority (95–98%) of deaths occur in developing countries. In some countries, more than one in five children die before they reach their fifth birthday. Seventy percent of these early childhood deaths are caused by just five conditions—pneumonia, diarrhea, malaria, measles, and malnutrition—all of them preventable or treatable *(6)*.

What is the incidence of pediatric cancers, leukemia in particular, compared with these diseases? Population-based data on the incidence of leukemia and other cancers in the developing world are neither consistently available nor reliable, owing to underregistration and the absence of organized cancer registries *(7)*. The average annual age-standardized rate (ASR) per million children in developed countries ranges from 100 to 150 cases per million children per year *(8)*. Lower ASR rates have been reported for developing countries *(9,10)*. Assuming an average cancer incidence of 105 and 125 per million children per year in developing and developed countries, respectively,

Table 1
Comparative Annual Age-Standardized Incidence Rates (ASRs) and 5-Year Survival for Acute Leukemias from Selected Registries

	India				
	Bangalore	*Bombay*	*USA*	*UK*	*Australia*
ALL					
ASR (no./yr/10^6)	12.7	11.0	26.7	29.7	34.3
5-yr survival (%)	36	22	69	72	73
ANLL					
ASR (no./yr/10^6)	6.2	3.3	5.0	5.9	6.2
5-yr survival (%)	10	6	26	34	41

Data from refs. *9*, *10*, and *14–16*.

the absolute number of new pediatric cancers in 1990 (and 2000) can be estimated *(11)* as 152,000 (178,000) in developing countries and 33,000 (33,000) in developed nations *(12,13)*. Of these, about one-third represent ALL, i.e., 50,000 (60,000) and 11,000 (11,000) in developing and developed nations, respectively. This is a huge burden.

The 5-yr survival rate for ALL in the United States is approx 75%, whereas rates from some of the developing countries range from 25 to 35% (Table 1) *(9,10,14–16)*. Thus, about two-thirds of children with leukemia in the developing countries, or 30,000–40,000/yr can be expected to die of their disease. Compared with the other common causes of death in children, acute leukemias do not constitute a highly significant proportion of children who die in developing nations. For example, 33,000 children die every day from infectious diseases, and a similar number probably die each year from leukemia. Hence, managing children with leukemia cannot be expected to be a health priority in developing nations. Nevertheless, we must recognize the needs of the individual child with leukemia and the impact of these requirements on the available resources and facilities. *The absolute numbers of such children are far too high to be ignored.*

3. ALL: SCENARIO IN UNDERPRIVILEGED NATIONS

As pointed out above and in Table 1, the overall survival rates in ALL are extremely poor in general in developing countries in comparison with those in the West. With the use of intensive chemotherapy protocols, some of the major centers in developing nations have recently reported improvements in survival rates *(17–20)*. There are several reasons for the lower survival rates: limitations in access to health care, limitations in the existing health care systems, factors related to general health and the social demographics of the patients, and differences in the biology of ALL *(21–24)*.

3.1. Limitations in the LES Nations: Financial Resources

Recently, the developing countries have been referred to as "less economically sound or privileged (LES) "nations". This phrase has merit because, although poor by western economic standards, these countries are rich in human and cultural resources *(25)*. Given the allocation of already scarce assets to health care and other pressing health needs such as family planning, nutrition, and prevention of infectious diseases, the task of managing children with leukemia seems herculean. The magnitude of resource constraints is apparent when one is confronted with the vast difference in the ratio of health expenditure allocation to the cost of treatment of a child with ALL. In a developing country such as India, the total government expenditure on health care per person per year is about U.S. $5, whereas that provided by the private sector is U.S. $16. [These figures were $2 and $6, respectively, in 1989 *(26)*] If one adds the cost of treating a child with ALL on a moderately intensive chemotherapy protocol for 2 yr, these costs escalate to U.S. $500–U.S. $2000, depending on whether the child is treated in the government or private sector. Obviously, the resources are just not available for managing children with leukemia.

In addition, in the developing world, resources are not evenly distributed, and the different strata in the population based on socioeconomic criteria must be considered when planning pediatric oncology services *(27)*. In India, 850 million (150 million households) of the entire 900 million population (95%) earn less than U.S. $1000 per annum *(28)*. Of these, over 350 million people (60 million households; one household = 5.5 people) earn less than one-third of this amount and live below the poverty line. Also, this lowest segment of the population is likely to increase greatly in the coming years. Chandy *(29)* has proposed three profiles or categories of population with regard to income, educational status, and motivation to undergo treatment.

Profile 1: Illiterate, laborer parents with a family income of less than U.S. $20 per month and no motivation to obtain adequate treatment for a cancer-stricken child constitute 70% of the population of the developing world.

Profile 2: Literate parents with a monthly family income around U.S. $50–100 and good motivation to obtain treatment but a lack of necessary resources constitute 25% of the developing world's population.

Profile 3: Elite educated parents from cities with monthly family incomes above U.S. $1000 who can use their own resources for the best possible treatment constitute < 5% of the population in the developing world.

With capitalism and market forces ruling the world economy, the heterogeneity in the population with regard to income, educational status, and motivation is a uniform feature of all developing countries, with the proportion of the population in each of the above categories differing among such countries. In the coming years, economic constraints will dictate that fewer rather than more patients will receive treatment *(30)*.

3.2. Limitations in the LES Nations: Other Barriers

Other, nonfinancial factors join the woefully inadequate governmental and family monetary resources to hinder the availability of adequate health services in the LES nations *(31)*. The most prominent among these are the educational and sociocultural barriers. A low literacy rate of just about 50% among Indians, with women hitting bottom at 30%, inhibits awareness of disease in general and leukemia in particular. Dissemination of information, efficient communication, and education of the masses is consequently slow and difficult. Compounding economic backwardness and lack of education are social mores and traditional cultural beliefs. These form major deterrents to

proper community utilization of health services. Motivation of the family to obtain treatment for their leukemic child is not always forthcoming *(32)*.

A combination of the above realities results in a majority of patients reaching treatment centers when the disease has reached an advanced stage, thereby minimizing the chances of administering curative treatment. Poor compliance with the treatment prescribed is the other consequence of the interplay of the above financial and social factors and accounts for the overall poor survival. The prohibitive cost of chemotherapy and supportive care is a major reason for poor compliance. Prolonged duration of treatment for the disease and its complications results in loss of earnings and family dislocation and also demotivates the parents. Meeting these complex social challenges is not easy and certainly cannot be done by medical personnel alone. The LES nations have to adopt a behavioral, social, and economic mode of change and uplift.

An important factor that prevents the administration of intensive chemotherapy as prescribed in the West is the relatively lower nutritional status of children, with consequent difficulties in tolerating intensive therapy. Nutritional factors and prevalent infectious diseases are likely to be among the most important environmental factors influencing the outcome and survival of children with ALL in LES nations *(33–36)*. Nutritional status of the child undergoing chemotherapy is associated with higher numbers of toxic deaths: patients below the median heights and weights for age had a significantly greater risk of dying from toxicity *(37)*. A high prevalence of hepatitis B viral infections among oncology patients in developing countries warrants a special mention: it leads not only to greater morbidity and toxic deaths but also unmeasured effects on survival from repeated interruptions in treatment *(35,36)*.

3.3 Differences in the Biology of ALL

Worldwide, ALL is the most common neoplasia. In developed countries, the incidence of ALL is >25 per million in children younger than 14 or 15 yr, and the incidence curve shows a peak between 2 and 5 yr due to a high incidence of common (CD10-positive) ALL *(38,39)*. In developing countries, the incidence of ALL is <25 per million (in low-income countries $<10/10^6$), the percentage of T-ALL is higher, and there is no peak incidence between 2 and 5 yr *(40–43)*. Moreover, ALL in developing countries is more often associated with lymphadenopathy and splenomegaly. In an Indian study reported by Advani et al. *(37)*, the presence of lymphadenopathy was significantly associated with a worse event-free survival (EFS). In their series, age and white blood cell (WBC) count at presentation proved inadequate to define risk groups, suggesting that prognostic factors may vary in different world regions. For 245 patients with a WBC count < $60{,}000/mm^3$, the most significant risk factor was the presence of lymphadenopathy (53% vs 77% EFS).

Differences in environment and lifestyle, as well as potential genetic differences, may influence not only the incidence of ALL, which appears to be threefold lower in India than in United States, but also its biology: T-cell ALL, for example, is more frequent in India, perhaps because of the relative paucity of precursor B-cell ALL *(44,45)*. Such differences in biology can influence both disease extent and the results of therapy. Acute nonlymphoblastic leukemia (ANLL) occurs at an incidence rate of 4–10 per million and is not related to socioeconomic status. In LES nations, chloromas are found in up to 30% of all cases with ANLL.

These differences in the biologic features indicate that in order to improve the survival of patients with ALL in developing countries, it is of utmost necessity to conduct research into the biology, response to treatment, and prognostic factors in the developing countries themselves *(37)*.

4. LEUKEMIA MANAGEMENT IN UNDERPRIVILEGED COUNTRIES: WHICH WAY TO GO?

There are two fundamental aspects to better management of leukemia in LES nations: one is the provision of easily available and affordable comprehensive pediatric oncology services for leukemia treatment, and the other is ensuring proper community utilization of these services or, in other words, increasing the access to care for the large majority of children with leukemia. Making the treatment affordable is a major hurdle. It is clear that many LES nations simply cannot afford to provide the care required for all newly diagnosed cases of leukemia. What are the strategies that are likely to succeed in such a grim situation? The definition of minimal requirements to guarantee effective treatment and the enhancement of the number of children with cancer who have access to treatment are the main goals *(11)*.

4.1 Establishment of Pediatric Cancer Units

The establishment at university hospitals or cancer centers of pediatric cancer units (PCUs) is the most efficient way to concentrate specialists; increase interdisciplinary cooperation; make optimal use of resources; develop supportive care; and ensure training, data management, and follow-up *(46,47)*.

A characteristic PCU model (with required staff, facilities, and resources obtained from the state, insurance, or private sector) for ensuring a favorable outcome in a child with cancer is depicted in Fig. 1. A PCU consists of at least a full-time pediatric oncologist and specialists willing to cooperate within the frame of the PCU. PCUs are essential for providing optimal pediatric oncology care for children and adolescents with cancer within the framework of available resources and for the training of pediatric oncology specialists at all levels.

In developing countries, PCUs can function efficiently without sophisticated imaging facilities, intensive care units, and hematopoietic precursor cell transplantation, provided they have a good hematology, microbiology, and histopathology laboratory; good surgical and radiation facilities; dedicated oncology nursing personnel; the necessary drugs and blood products; and minimum facilities for data management and follow-up. Standardization (adjusted to the local conditions) of initial investigations, treatment, supportive care, and evaluation of toxicities and treatment results is essential in order to make progress *(11)*. The limitations of supportive care have to be kept in mind when decisions regarding the aggressiveness, the complexity, and the duration of treatment are made. Hence, the PCUs in developing countries have responsibilities to establish "standards of care":

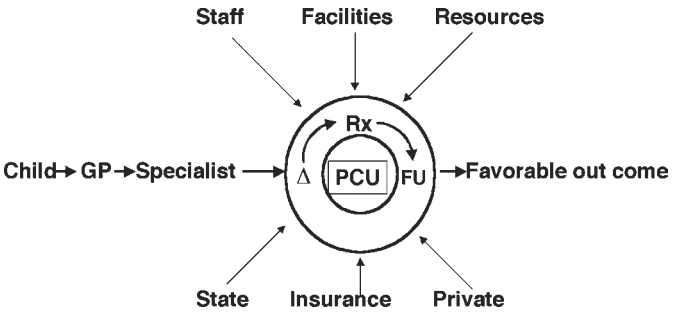

Fig. 1. Pediatric cancer unit (PCU) model for the provision of comprehensive pediatric cancer care. GP, general practitioner; Rx, treatment; FU, follow-up; △, diagnosis.

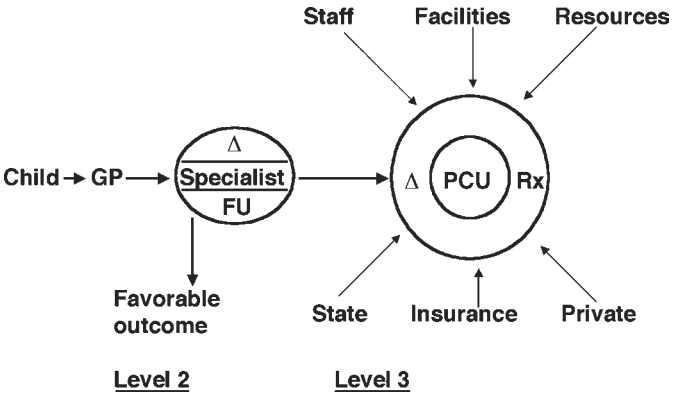

Fig. 2. Shared-care pediatric cancer unit (PCU) model. This strategy would help to ensure early referrals and early diagnoses, as well as adequate follow-up care of children with leukemia in developing countries. FU, follow-up; GP, general practitioner; Rx, treatment; △, diagnosis.

minimum diagnostic criteria, tailored effective protocols that are both affordable and practical, and structured supportive care guidelines relevant to the existing situations.

4.2 Expanding Access to Health Care: Shared Care Model

Pediatricians practicing in developing countries have to confront the challenge of making leukemia treatment accessible to all those in need. This challenge can be met by working collaboratively with the policy makers and the community and by reorganization of existing services through a tight linkage of primary, secondary, and tertiary care services. A shared care model for developing countries (Fig. 2), with the involvement of pediatric specialists at a district level, whether in the governmental or private sector, would ensure early referrals and early diagnosis, as well as participation in follow-up care of children diagnosed with leukemia. The concept of shared care with peripheral participation would be beneficial to both the patients and the crowded hospitals with pediatric cancer units. Decentralization of the treatment process by a shared management approach would decrease the workload on the PCU, increase the percentage of children with cancer who have access to treatment, and be well accepted by the patients and their parents because of decreased direct and indirect costs (traveling expenditures, time, and loss of wages). This is very important for poor families who have no "buffer funds." By training and exchanging staff with peripheral hospitals and dispensaries and by establishing contacts with practitioners, PCUs could contribute to earlier detection and referral of cancer patients. Thus, sharing of leukemia treatment responsibilities would make therapy available to all children and minimize some of the logistic, economic, and psychological problems experienced in implementing centralized treatment based on a PCU model.

The most efficient approach would entail a national plan whereby resources are used optimally and an organized approach is developed for training the required professionals, identifying regional sites for pediatric oncology units, and providing appropriate diagnostic know-how and treatment protocols tailored to the specific needs of the country. Pediatric oncology services in developing countries need to be reorganized utilizing the existing health care infrastructure, as shown in Table 2. The Indian health care infrastructure is used as prototype for demonstration purpose.

4.3. Education of Professionals and Community

If pediatric oncology is to develop optimally and if care is to reach appropriate high standards, a training program for health care professionals of all categories and the community at large must be established as a priority. Many people in India, including some physicians, still feel that cancer is not curable and that treatment of children with this disease is not worthwhile. Since these people form a majority, their advice influences the parents of patients, who all too often decide to obtain minimal symptomatic treatment locally without "wasting" many of their scarce resources. The education of professionals as to the possibility of permanent cure of malignant diseases is certainly a priority. A well-planned global professional educational program needs to be directed toward pediatricians, pediatric surgeons, nurses, and ancillary paramedical staff, who form the vanguard in the fight against pediatric cancer. Increased awareness of the community through various educational activities would also make more parents bring their children in earlier for cancer treatment, thereby improving survival.

4.4. Twinning Programs

Twinning is a global, long-lasting cooperation between a pediatric cancer center in a developing country and a center from a developed country. The training of PCU personnel in underprivileged nations is best promoted by this strategy. The La Mascota ALL twinning program between La Mascota Pediatric Hospital, Managua, Nicaragua, and hospitals in Monza and Milan, Italy, has clearly demonstrated how intellectual, organizational, and financial resources can be generated by a dedicated twinning program. It is vital to the success of such a program to have a long-term commitment to a comprehensive and holistic strategy that incorporates the supply of drugs, the training and supervision of health professionals, and the care of childhood cancer patients and their parents *(48)*. Masera et al. *(48)* have pointed out that cooperation in twinning was not simply an exercise of transferability of established diagnostic and therapeutic protocols, but rather part of a research effort aimed at adaptation and assessment of the protocols in the specific conditions of Nicaragua.

Table 2
Levels of Care: Guidelines for Pediatric Oncology Practice.
Organization of Services with Distribution of Responsibilities
Within the Existing Health Care Infrastructure of Developing Countries (Indian Model).

Level of Health System		*Responsibilities*	
Facility	*Unit*	*Lab services*	*Therapeutics*
Regional cancer center	PCU	IP/IHC, molecular assays	Chemotherapy, radiotherapy, BMT
Medical college, Dept. of Pediatrics	PCU	Hematology, imaging, histopathology	Initial chemotherapy, blood components
District headquarters hospital	Outreach center	CBC, biochemistry, microbiology	Chemotherapy with follow-up

Abbreviations: PCU, pediatric cancer unit; IP/IHC, immunophenotyping/immunohistochemistry; CBC, complete blood count; BMT, bone marrow transplantation.

One of the lessons of the La Mascota program is the demonstration that the gap between existing resources and their availability where they are most needed can be bridged *(49)*. The responsible transfer of effective interventions (e.g., for treatment of ALL) cannot wait for future unspecified economic developments in the developing world; in the absence of effective methodologies such as twinning, resources for expensive advanced interventions will inevitably be restricted to the few who can pay for them. In 1997, the International Society of Pediatric Oncology (SIOP) adopted the Montevideo statement to promote the systematic implementation of center-to-center cooperation as a concrete contribution to the establishment of pediatric hematology–oncology programs in developing countries *(50)*. A word of caution for the underprivileged LES nations: although twinning will pave the way, individual countries must find their own solutions to the problem of resource generation, independently of the West.

4.5. Role of Therapeutic Alliances

The most relevant, crucial limitation to the progress of pediatric oncology care in developing countries is the lack of resources. This situation has worsened over the last few years (and will worsen further in the years to come) due to the increasing cost of health care services, economic restrictions in most countries, increasing foreign debt, and programs of the World Bank and the International Monetary Fund *(51)*. The most promising opportunities for tackling this resource crunch come from groups of parent-volunteers and their cooperation with pediatric oncologists and other health professionals in the context of the "therapeutic alliance." This is a realistic means to sensitize society and mobilize resources and energies at national and international levels. The most productive way for underprivileged LES nations to generate resources is to motivate the parents of children with cancer to support pediatric oncology. Strong and viable parent organizations or support groups are essential when resources are scarce, parents are disempowered, and the psychosocial needs of families are unmet.

This methodology has worked in many situations around the world for pediatric cancer as well as other pediatric illnesses, for example, the therapeutic alliance program in Monza, Italy, with the Tettamanti Foundation and the parents' association Comitato ML Verga. GRAACC, a support group for children and adolescents with cancer in São Paulo, Brazil, in collaboration with the University of São Paulo and community volunteers, parents, and the physicians *(52)* was successful within 3 yr in constructing a modern 11-floor pediatric oncology hospital for supporting more than 200 low-income new pediatric oncology patients each year. The creation of a therapeutic alliance between the doctors and the community through the patients/parents may be an important option to improve funding for pediatric oncology in developing countries and may have a major impact on early diagnosis, medical care, and ultimately patient survival.

4.6. National Cooperative Groups

Creating cooperative groups and conducting clinical trials to answer specific scientific questions have been the cornerstone of success in pediatric oncology throughout the world. The collaboration of all PCUs of a country, in a national cooperative group that participates in research projects to answer scientific questions of relevance to pediatric oncologists in the developing world, is to be considered a desirable priority, and one of the minimal requirements for pediatric oncology practice in the developing world. Answers to difficult questions, such as the prognostic factors of importance in risk stratification of childhood ALL patients, must be found by practitioners in the developing countries themselves.

5. SPECIFIC ISSUES IN THE MANAGEMENT OF CHILDHOOD ALL

Developing countries must make every effort to provide the best possible diagnostic and treatment facilities for all children with ALL, irrespective of their socioeconomic status. However, the reality of resource constraints dictates otherwise. In the absence of adequate state support and given the heterogeneous economic resources available to families, decisions regarding the application of simple vs intensive treatment protocols to be used for the individual child with ALL are extremely difficult and challenging. On the one hand, with simple protocols, the resultant survival rates may prove to be unacceptable; on the other hand, more aggressive protocols may prove too demanding in terms of the resources required for intensive supportive care.

5.1. Minimal Requirements for Diagnosis

What should the minimal standards be for establishing the diagnosis of leukemia in children of developing countries? Is a

combination of morphologic and cytochemical assessment sufficient? What should be the role of immunophenotyping? The role of molecular genetics? We would consider good morphologic evaluation with cytochemical assessment, done at the earliest opportunity in a child suspected of having leukemia, to be a minimal requirement. Examination of the cerebrospinal fluid by cytospin for the presence of blast cells and chest roentgenography to detect mediastinal enlargement would also be necessary. The percentage of children identified as high risk on the basis of immunophenotypic or molecular markers, but not identified by clinical criteria (e.g., age, lymphadenopathy, organomegaly) or leukocyte count at presentation, would probably be very small.

We also have difficulty in applying aggressive leukemia protocols in the treatment of a large proportion of children with high-risk features. Hence, before we embark on molecular biologic studies, we must at least make sure that a simple primary diagnosis is rapidly made and that facilities for such procedures are available and readily accessible. For the profile-3 patients who can afford the costs, such facilities should be made available, at a price, at a few pediatric cancer centers. This approach may benefit some of the profile-1 or profile-2 patients, for whom the costs of these investigations could be subsidized whenever necessary. Hence, establishing the diagnosis of ALL in developing countries is not very demanding and could easily be done at the health care facilities of a district hospital. There is a need for a high level of clinical suspicion and immediate evaluation of the peripheral blood in cases with suggestive signs of leukemia, so that the children are referred early to PCUs.

5.2. Treatment Options and Elements in the Management of ALL

The cornerstone of modern ALL therapy has been the careful assessment of failure risks, so that high-risk cases are treated aggressively and less toxic therapy is reserved for lower-risk cases. It must be pointed out that of the many variables that have been linked to ALL prognosis, treatment has emerged as most influential: two examples are T-cell and B-cell ALL, which were once associated with a dire prognosis but now have long-term response rates of 70% or better with the use of intensive chemotherapy *(53–57)*. Age and leukcocyte count at presentation were recommended by a National Cancer Institute workshop as minimal criteria to define low-risk ALL (age between 1 and 9 yr and a leukcocyte count of $<50 \times 10^9$/L). Whether these criteria and definitions hold true for populations in developing countries remains to be determined. Because patients often present with advanced disease, it is possible that there will be a greater proportion in the high-risk category. To start with, uniform assessment criteria must be used by pediatric oncologists treating children with ALL in developing countries, so that the importance of different prognostic variables becomes clear.

The major components of contemporary protocols for childhood ALL include agents for remission induction, intensification/consolidation, prevention of overt central nervous system (CNS) disease, and continuation of remission *(58)*. The recipe varies from center to center, and it must be remembered by pediatric oncologists in developing countries that importing and using protocols from the West may not be cost-effective or even critical, so that particular elements of the protocol must be considered carefully. Examples abound: use of dexamethasone, vincristine, and anthracycline without asparaginase in remission/induction to decrease costs *(59)*, continued triple intrathecal therapy in standard risk ALL in lieu of CNS radation *(60)*, and use of vincristine and dexamethasone pulses during maintenance therapy to decrease the risk of CNS relapse *(59)*. Such information should be optimally utilized to design tailored protocols for individual patients. At present, there are no standard protocols that can be uniformly recommended for use in the developing countries.

The other consideration in deciding on a treatment strategy for childhood ALL in developing countries is the socioeconomic profile of the individual child and her or his family. An approach based on these profiles has been proposed by M. Chandy (Table 3). It provides a structural backbone for decisions regarding the treatment options available to a pediatric oncologist for the treatment of childhood ALL in a developing country.

5.3. Requirements for Supportive Care

The facilities for providing supportive care are often lacking in the developing world; hence, toxic deaths related to aggressive chemotherapy are a common occurrence *(61–63)*. Another disturbing factor is the high levels of antimicrobial resistance in developing countries where sanitation is poor, which contribute to higher rates of fatal sepsis *(64)*. Facilities for blood transfusions are inadequate, and blood components are available only in a few tertiary centers. Hence, the institution of aggressive chemotherapy or the choice of a chemotherapeutic protocol must be based on the available supportive care services and facilities. Improved supportive care can reduce mortality caused by infection remarkably and can improve disease-free survival rates as well *(65)*.

6. ETHICAL AND RESEARCH ISSUES

There is a considerable gap between the exciting scientific developments in the field of medicine and what is readily available to the underserved segments of society *(66)*. It is necessary for the world community to realize the vital role of social and environmental factors in determining both the disease pattern and the approach to health care in developing countries. Hence, effective but low-cost scientific interventions need to be developed separately for the developing world in order to achieve universal success. With regard to ALL management, the ultimate goal should be to treat every child worldwide with curative intent. The dilemma comes when resources and facilities are meager. One option is to allocate resources on the basis of presumed maximum benefit. But can one deny adequate treatment to a 4-yr-old girl with B-cell precursor ALL for want of sufficient resources? It's a difficult choice but one that is made every day by parents and physicians taking care of children in underprivileged nations. Legislators and health authorities in such societies have more clear-cut choices: to focus their efforts on general measures that are known to be effective, such as immunization and sanitation *(67)*. These relatively inexpensive improvements will benefit far more children by maintaining their good health, rather than expending similar sums in the

Table 3
Strategy for Treating Childhood Acute Leukemia in India

Profile[a]	*Risk status*	*Regimen*	*Author's comments*
1	Low	VCR, PRED induction, IT MTX, 6MP, MTX	Simple national protocol implemented in a cooperative trial (with provision of drugs) can be used to answer scientific questions
	High	Analgesics, steroids	Difficult to tackle
2	Low	UKALL VIII	Intensification blocks beneficial
	High	BFM 76/79	Similar moderately intensive protocols can be applied
3	Low	BFM 76/79	Many alternatives available
	High	BFM 90	Many intensive chemotherapy alternatives available

Abbreviations: VCR, vincristine; PRED, prednisone; IT MTX, intrathecal methotrexate; 6MP, 6-mercaptopurine.
[a]See text for definitions.
Modified from ref. *(32)*.

attempt to restore health to a few. These issues must be faced and solved by the developing nations themselves; there are no easy solutions that can be advocated.

Many difficult questions must be answered by the developing countries before they can embark on new clinical and social research concerning the management of ALL in children. The answers must be sought with properly designed research protocols. Which prognostic factors predict high-risk ALL and a poor outcome? What is the socioeconomic risk profile at presentation in our population? What is the minimal but effective therapy that we can afford to give our patients within the economic and logistic constraints of our infrastructure? Is CNS irradiation necessary for all children or could we do without it? What are the compliance and dropout rates among the different socioeconomic profiles? Because genetic, ethnic, nutritional, and other factors may affect drug delivery, pharmacokinetics, and toxicity profiles, what are the pharmacokinetic, and toxicity profiles of drugs used in ALL therapy in our population? The challenge lies in the commitment of colleagues in developing countries to seek answers to these questions in a cooperative framework.

7. CONCLUSIONS

The treatment of ALL in underprivileged populations is beset with multiple hurdles. Leukemia is not a high priority compared with the infectious illnesses afflicting children from these nations. However, there are five times more children with acute leukemia in the developing nations than there are in the developed nations, and the expected clinical outcome of these children is deplorable. Given the meager resources and other constraints in these countries, together with the apparent differences in the leukemia biology, immediate relief or hope does not seem to be in sight. The scenario could improve in the coming years. Small but steady and consistent steps, as outlined in this article, are capable of bringing about a dramatic change in the management of ALL in underprivileged children from the developing world.

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24 How to Confront the Problem of Treatment of Childhood Leukemias in Underprivileged Populations

GIUSEPPE MASERA AND ANDREA BIONDI

CONTENTS

1. INTRODUCTION

The improvements achieved over the last 50 yr in the field of childhood cancer research have generated conflicting results. As shown in Fig. 1, the survival of affected children has increased more than threefold, to about 70–75%, in the developed countries. These results have been achieved by concentrating specialized personnel in pediatric care units (PCUs), where optimal treatment is provided and there is rapid transfer of dramatic advances in clinical medicine, pharmacology, biology, radiotherapy, and new technologies *(1,2)*. Unfortunately, only 20% of all children with cancer can obtain access to adequate treatment. For the remaining 80%, about 180,000 children/yr, the treatment is generally inadequate or totally impossible, as demonstrated by an inferior 5-yr survival rate, about 20–25%.

The difference in survival between children living in developed and developing countries (North and South) is a reminder of a wider truth: the asymmetry between the global regions, with respect to the basic right to life, is at the same time both total and avoidable *(3)*. Childhood cancer in low-income countries (LICs) is becoming a problem of critical importance and deserves appropriate intervention besides the more traditional measures taken for malnutrition or acute respiratory or diarrheal diseases *(4)*. The relevance of the problem becomes clear from the following considerations:

1. Eighty-four percent of all children younger than 15 yr old lived in developing countries in 1990 *(1,5)*. Assuming an average incidence of 105 and 125 cases (per million) per year in developing and industrialized countries, respectively *(1)*, one can estimate the absolute number of new pediatric malignancies. In the year 2000, 177,000 childhood cancers have been referred to be diagnosed in countries of the South and 33,000 in the North *(1,5)*. The former number is likely to increase further by 2030, when 90% of the word's childhood population is expected to live in LICs.
2. The decreased mortality from infectious diseases has underscored the importance of noncommunicable diseases (including cancer), even in countries of the South. As in the North, among children 5–14 yr old, cancer is becoming a more relevant cause of death with improvements in control of measles, diarrhea, and pulmonary infections.
3. Together with the more relevant role of cancer, the costs of delivering adequate treatments have dramatically increased, further reducing the possibility that countries with a weak economy will be able to address the mounting needs of their social and health programs. As a result, the inequality gap between North and South is likely to widen further. The health of children has therefore become an indicator of the growing inequality between developed and low-income countries and of the human costs of economic development *(6–8)*.

In the last few years, several institutions or organizations in the North have promoted a number of initiatives around the

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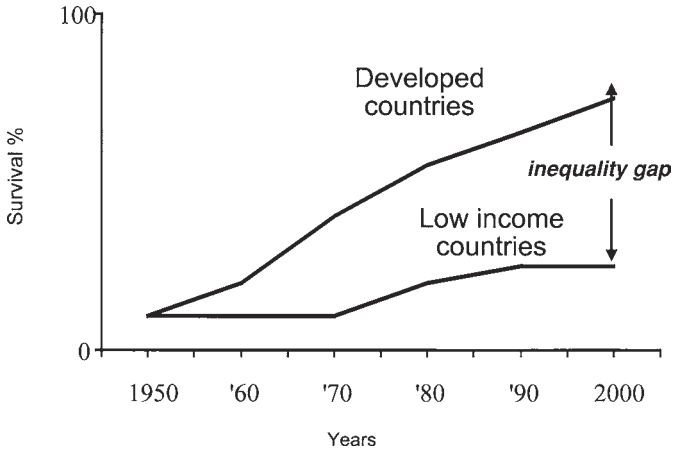

Fig.1. Survival of children with cancer (Adapted from Ref. *22*).

world in an attempt to ensure that all children have access to reasonable care for leukemias and other cancers. A consensus document was presented at the XXVII Meeting of the International Society of Pediatric Oncology (SIOP), held in Montevideo in 1995. This document (The Montevideo Statement; Table 1), which proposes that each advanced center of the North should consider becoming a twin of a center in the South, was approved by SIOP in 1997 *(9)*. More recently, a new organization, the International Network for Cancer Treatment and Research (INCTR), started its activities *(10)*. This international nonprofit organization, registered in Belgium, is dedicated to helping patients with cancer in countries with limited resources. The INCTR will assist developing countries through a structured program of research collaboration, education, and training *(10)*.

If the intervention of international agencies is absolutely necessary, it is difficult to foresee concrete change without an active assumption of responsibility by pediatric oncologists together with others (parents, nurses, volunteers, and others) affected by the challenge of a child with cancer, all of whom must collaborate to mobilize resources, public opinion, and the endorsement of authorities by demonstrating the feasibility of the proposed actions. Adoption or linkage-twinning should not be assumed as an ethical commitment but rather practiced as a natural consequence of professional awareness and expertise.

In this regard, a twinning program between La Mascota Pediatric Hospital in Nicaragua and the Pediatric Hematology and Oncology Center in Monza, Italy, was set up in 1986. The program of teaching self-help resulted in a well-established pediatric oncology service in an area where once there was very little *(11,12)*. To promote and articulate this perspective further and to symbolize the evolution of the twinning idea in its 10th anniversary, the Monza International School of Pediatric Hematology-Oncology (MISPHO) was launched in 1996 *(13)*.

The aim of this review is to discuss some modalities of intervention that may improve the outlook for pediatric hematology–oncology in developing countries and may prepare these nations better to confront the problem of treating childhood acute lymphoblastic leukemia (ALL) in underprivileged populations.

Table 1
Extracts from the Montevideo Statement

The current situation

- When adequately treated, 60–70% of childhood cancers are currently curable.
- Today only 20% of the children worldwide benefit from adequate state-of-the-art therapy.
- 80% of the world's children, i.e., those in the countries with limited resources, are denied a cure.
- Cancers represent the first disease-related cause of death among children in developed countries (second only to accidents).
- Mortality from childhood cancer is the third or fourth cause of death among children in many of these developing countries.
- Childhood cancer is increasingly important in countries with limited resources, because of the substantial increase of children in the population as a result of the high birth rate and the decreased mortality from communicable diseases or malnutrition.
- Mortality from childhood cancer could be reduced by the establishment of pediatric hematology-oncology programs with centers that provide free essential therapy to all children who need treatment.
- The implementation of such programs should be considered a step toward the application of the United Nations Convention on Children's Rights (New York, Nov. 29, 1989).

We therefore request the governments and international financial bodies:

- To take into consideration the issue of pediatric oncology in countries with limited resources.
- To undertake initiatives and allocate resources for the implementation of pediatric oncology care and research programs.

As representatives of the family of pediatric oncology, we commit ourselves, and we propose:

- To foster all activities that may contribute to the mobilization of the community at large toward programs of international cooperation.
- To promote the systematic implementation of center-to-center cooperation as a concrete contribution to the respect of the neglected rights of children.

From ref. *12*.

2. STRATEGY FOR THE ORGANIZATION OF A PEDIATRIC ONCOLOGY PROGRAM: LESSONS FROM A NORTH-SOUTH TWINNING PROGRAM

The idea of introducing ALL among the traditional targets of cooperation was based on the hypothesis that the adoption of such a disease could not only become a challenging way to tackle preventable deaths but could also symbolize hope in a

disheartening situation. The project was developed by a comprehensive disease approach that included training, supply of basic resources, and strengthening of health care facilities. The training of a core group of medical doctors and nurses was the first step of the project, which was made possible by private financial support centered around the two clinical groups involved (Monza, Italy and Bellinzona, Switzerland). A striking result of the program was that all people so far trained returned to and still work at La Mascota Hospital, despite the unstable economic and institutional conditions in Nicaragua *(11,12)*.

As soon as ALL could be diagnosed and induction treatment started, it became clear that one of the major obstacles to the successful implementation of the program was the absence of minimal structures for the provision of adequate care in the hospital, for lodging the parents of children, and for outpatient care. (Logistic and economic difficulties prevented scheduling of regular visits to the hospital.) The construction of a PCU consisting of two clean pavilions (10 beds each for hematologic diseases and solid tumors), of an outpatient clinic, and of a parents' house was made possible through the support of charities in Italy and Switzerland.

The solutions adopted to tackle the difficulty of family participation in the process of care are an interesting result of the program. Our approach was tailored to the living conditions of each child, which were assessed with the support of a social worker, and incorporated the length of hospital stay, use of the parents' house, and the need for and provision of money for transport to ease the delicate tension between the long absences of a parent and the isolation of the child from the family. Financial support to promote greater access to the PCU, thus avoiding treatment refusals or early abandonment of the program, was awarded to children with more severe social and poverty problems. Refusals or abandonment of treatment are both cultural problems, linked to the recognition by the community of the likelihood of cure, and to economic factors such as the costs of therapy (drugs, travel to reach the treatment center, and so on).

Eventually, it became clear that the interests and competence of the staff had expanded gradually to include most forms of childhood cancer. After 14 yr, >130 children with cancer are referred, diagnosed, and treated every year at La Mascota Hospital *(12)*.

Overall, a twinning program should be conceived and promoted from the start as a research project, with well-defined rules (Table 2) with which all participants must comply. In one sense, a twinning program can be compared with marriage: it requires a long-term commitment and strong motivations that should characterize the behavior of the different partners.

The methodology of "twinning" has been successfully applied in other situations around the world for pediatric cancer as well as for other pediatric illnesses. The St. Jude Outreach Program developed several partnerships around the world based on five components: involvement of local leaders, evaluation of resources and needs, training of key personnel, financial support for critical operational needs, and implementation of standardized therapy. Successful treatment of childhood ALL has been achieved in El Salvador, in the context of a partnership program started in 1994 *(14)*, with a 4-yr event-free survival rate of 46% in the high-risk group and 69% in the standard-risk group (n = 153 overall).

Table 2
Rules for a Cooperative Twinning Program

- Encouragement of individuals from both sides who are seeking change or reciprocal promotion and autonomy, to take part in the project.
- A comprehensive disease-oriented approach that includes training, supply of basic resources, and strengthening of structural facilities.
- Long-term planning (minimum 5 yr), with periodic reassessments and adjustments of strategies and needs.
- Clearly defined and progressively implemented policy of transfer of management responsibilities to local professionals.
- Cooperative adaptation and tailored adoption of the most important international protocols and guidelines, as teaching and planning tools.
- Financial support (in the donor pool) from diverse sources to permit independence and flexibility of the project.
- Early involvement of community and parents' representatives to allow effective identification of needs and priorities.
- Ensuring transparency of all project activities, with periodic reporting to supporting groups.
- Establishment of research projects (with ad hoc financial support) to motivate the professional groups further and encourage broader cultural interest.

From ref. *12*.

2.1. Role of Therapeutic Alliances

A major resource in promoting and supporting pediatric oncology programs consists of the close collaboration (alliance) of the medical staff with the parents of children with cancer and with the volunteers. This aspect of the relationship between physicians and parents has been well analyzed in a document recently elaborated by the Psychosocial Committee of the SIOP *(15)*.

Such "therapeutic" alliances generally entail family members-parents, the patient, and the medical staff, all working toward a common goal: curing the cancer and minimizing its medical and psychosocial side effects, as well as mobilizing the energies of all members of the society to this end.

The diagnosis of cancer in a child produces sudden and overwhelming challenges for the family. Beginning with the initial phase of fear, shock, and disbelief, most primary coping patterns involve the mobilization of intense energy. Depending on personalities and circumstances, these energies can express themselves in denial, anger, and belligerence or, alternatively, in passivity and withdrawal. A compulsion to learn about the disease and its treatment, to do everything possible for the child, and a wish to collaborate actively in medical care coexist interchangeably over time and are displayed differently by family members.

The will of the parents, child, other family members, and friends to fight the disease, together with staff, expresses itself in each country and each economic class through the desire to

gather in associations or committees. At the base of this phenomenon, there is the wish to discuss the sense of isolation and frustration caused by the disease, the difficulties and the limitations of therapy, and need to support research.

In pediatric oncology, parents' associations are common worldwide and act either locally or at national and international levels. The International Confederation of Childhood Cancer Parents Organizations (ICCCPO) is fully dedicated to such efforts. This strategy is important in countries of the North, but even more so in countries of the South, where resources are scarce and the economic and psychosocial needs of families cannot be easily fulfilled.

2.2. Research Opportunities

One of the major challenge in a twinning program is to ensure that cooperation does not simply result in the transfer of established diagnostic and therapeutic protocols but rather promotes research aimed at adaptation and assessment of the protocols in a specific cultural and economic condition. In our experience in Nicaragua, as soon as the minimum requirements of quality of routine care were ensured, the Nicaraguan team was fully involved in more formal research projects that led to the joint authorship of scientific papers *(16–19)*. Thus, several questions pertinent to this issue can be raised. What research is relevant for LICs? What interests might more developed countries have in such research? And what are the roles and responsibilities of journals in the North in providing information on such research? The transferability of therapies, a critical, evidence-based assessment of what is essential, and the cost-benefit profile of any proposed research are not only fundamental aspects of oncology in the South but are also important in the North *(20)*.

Jean Bernard pioneered the definition of *geographic hematology* to underline the differences between the various populations in the expression of a disease (*hématologie ethnique*) as well as the role of environmental factors in its pathogenesis (*hématologie péristasique*). By linking very different fields, through extremely varied disciplines (molecular biology, physiology of nutrition, parasitology, and virology), geographic hematology finds its unity in prevention and offers unique opportunities for our understanding of the pathogenesis of many blood diseases. Both of these approaches have provided unique opportunities to study the etiologic factors in many blood diseases *(21)*. We support the inclusion of *social hematology* in those perspectives originally defined by Bernard, to support all scientific efforts dealing with the application of the progress in countries with limited resources *(22)*.

3. ISSUES IN THE TREATMENT OF CHILDHOOD ALL

3.1. Can We Define an Optimal Treatment Protocol for LICs?

The optimal strategy for treating childhood leukemia in underprivileged countries cannot be devised superficially, for example, by adopting the protocol of one of the international cooperative study groups or, alternatively, by using a generic protocol for "developing countries." The treatment protocol should take into account, first of all, one's own experience, the available facilities, and a therapeutic objective that can be carried out realistically. A careful evaluation of success probabilities, the intensity of treatment, and the seriousness of infectious complications should avoid the adoption of overly intensive therapies that could produce high mortality rates. Furthermore, protocols should be based on all the evidence that is available internationally, acquired ideally not just in a literature search but also by direct contact with as many colleagues in the field as possible *(23)*. From that perspective, the use of twinning methodology can offer continuous and lasting support in consultation and a rapid transfer of recent results.

In experience with colleagues in Managua, Nicaragua, the drafting of protocols has been readily achieved by critical evaluation of each component of the therapy with respect to published data and transferability and finally by a realistic estimation of the expected cure rate (e.g., a 5-yr survival of 50–55%).

3.2. Can We Define the Expected Proportion of Children to Be Cured?

It is clear that the magnitude of resource constraints dictates success in treatment of children with cancer in LICs. Chandy *(24)* has proposed three profiles or categories of populations in India with regard to income, educational status, and motivation to undergo treatment *(24)*. Although this solution represents a concrete approach successfully applied to confront population heterogeneity in a center of the North, we would still strive to provide the "best possible" therapy that can be offered to all children with leukemia, regardless of their socioeconomic status. Undoubtedly, this goal could be considered utopian compared with the more realistic and pragmatic assessment that it is actually impossible to offer adequate treatment (even less intensive) to most children in LICs. However, the utopian strategy can produce satisfactory results when the different components of what we define as the childhood cancer family collaborate to mobilize resources both locally (alliance) and internationally (twinning or partnership).

3.3. Learning from Experience

A careful evaluation of the results achieved and the progressive identification of problems and possible solutions represent an essential element of the twinning strategy that requires the transfer of knowledge in collecting, managing, and evaluating data. In our ongoing experience in Nicaragua, mortality due to infections was an important cause of failure, at the beginning of the partnership. It was crucial to understand precisely the cause of those events and to adopt modifications in the treatment protocol and supportive care measures. Mortality caused by infection arising in the first days after the beginning of therapy is likely to be dependent on preexisting or concomitant factors (e.g., infections, malnutrition), thus requiring a specific strategy of intervention. By contrast, mortality caused by infection arising in the second part of the induction phase is probably related to the intensity of treatment, which should be tailored to the local conditions and the access to adequate antimicrobial therapy.

Another important factor that may affect prognosis is the refusal or abandonment of therapy early, in complete remission, a prominent consequence of educational and sociocultural barriers in LICs. In Nicaragua's experience, abandonment of treatment involved about 30% of cases at the beginning of the program. This rate dramatically decreased,

to 5–10%, with the inclusion of a program of individual adoptions, whereby each child with a new diagnosis and his or her family were adopted for treatment by a twin family from Italy or Switzerland. Furthermore, the construction of a residence near the hospital has made early dismissals possible, as well as fewer discomforts for the family during the more critical phases of treatment.

4. CONCLUSIONS

We quote the following Chinese saying, which was referred to us as a motto for a good teacher *(25)* but which can be applied to the spirit of cooperation: "Go in search of your people: love them, learn from them, plan with them, serve them; begin with what they have, build on what they know. But when their task is accomplished, the people will all remark, 'We have done it *ourselves'*."

It is realistic to say that North–South asymmetry will remain for a long time. It will probably increase and become a rule unless two conditions are met: (1) professional adoption (twinning/partnership) must become a widespread normal practice in most research groups in the North; and (2) research interests must be well served by the interplay resulting from North-South asymmetry *(26)*. Twinning programs and therapeutic alliances have already produced impressive results, by mobilizing intellectual, cultural, and financial resources. These achievements represent small but significant symbols of "globalization with a human face," at least for an important segment of the world's pediatric hematology-oncology community.

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ANTILEUKEMIC DRUGS

III

A

HIGH-DOSE METHOTREXATE IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

25 Rationale for High-Dose Methotrexate in Childhood Acute Lymphoblastic Leukemia

TIMOTHY L. BRENNER AND WILLIAM E. EVANS

CONTENTS

1. INTRODUCTION

During the 1950s, methotrexate replaced aminopterin as the cornerstone of antifolate therapy in the treatment of several malignancies, including childhood acute lymphoblastic leukemia (ALL). Today, the 5-yr event-free survival (EFS) rate for children with ALL exceeds 75%, and methotrexate is a component of essentially all childhood ALL chemotherapy regimens *(1–18)*. However, *de novo* or acquired resistance of leukemic cells to chemotherapy remains an obstacle to cure in the remaining 20–25% of patients. Giving high doses of methotrexate followed by leucovorin rescue is a widely used strategy to overcome resistance to antifolate therapy. To this end, methotrexate doses ranging from 20 to 33,000 mg/m^2 have been used in clinical trials, but the optimal dose for childhood ALL remains unknown. This chapter discusses the rationale for high-dose methotrexate (HDMTX) therapy in childhood ALL and reviews laboratory and clinical studies that provide insighßc into the appropriate dosage of methotrexate.

2. MECHANISM OF ACTION

To understand methotrexate cytotoxicity and resistance, it is important to consider its mechanism of action. The intracellular metabolism and effects of methotrexate are summarized in Fig. 1. This compound must enter lymphoblasts to be cytotoxic, and several influx mechanisms are utilized, including (1) the reduced folate carrier (RFC), a bidirectional anion exchanger; (2) the high-affinity low-capacity folate receptor (FR), which activates an endocytic process; and (3) passive diffusion. The K_m values for the RFC and FR are in the micromolar (μ*M*) and nanomolar (n*M*) ranges, respectively. Consequently, at typical methotrexate plasma concentrations in patients (1–20 μM), the RFC is considered the major mechanism of methotrexate influx, because the FR saturates at concentrations >0.1 μ*M* *(19)*, and passive diffusion is relatively minor until plasma concentrations exceed approximately 100 μ*M* *(20)*.

Once inside the cell, methotrexate either binds to its target enzymes, including dihydrofolate reductase (DHFR), or is polyglutamated by cytosolic folylpolyglutamate synthetase (FPGS) to methotrexate polyglutamates (MTXPG). As many as five additional glutamates may be sequentially added to the molecule at the 6-carboxyl end of glutamate *(21)*; however, methotrexate is polyglutamated at a slower rate than naturally occurring folates. Both methotrexate and MTXPG bind to DHFR to inhibit formation of tetrahydrofolate, a required cofactor for thymidylate and purine biosynthesis *(22,23)*. However, MTXPGs also inhibit key enzymes of *de novo* purine synthesis, primarily 5'-phosphoribosylglycinamide (GAR) and aminoimidazole carboxamide ribonucleotide (AICAR) transformylases *(22,24,25)*

$MTXPG_{4–6}$, containing more than three glutamyl residues, remain in cells for a longer duration and have higher affinities for target enzymes, compared with short-chain $MTXPG_{1–3}$.

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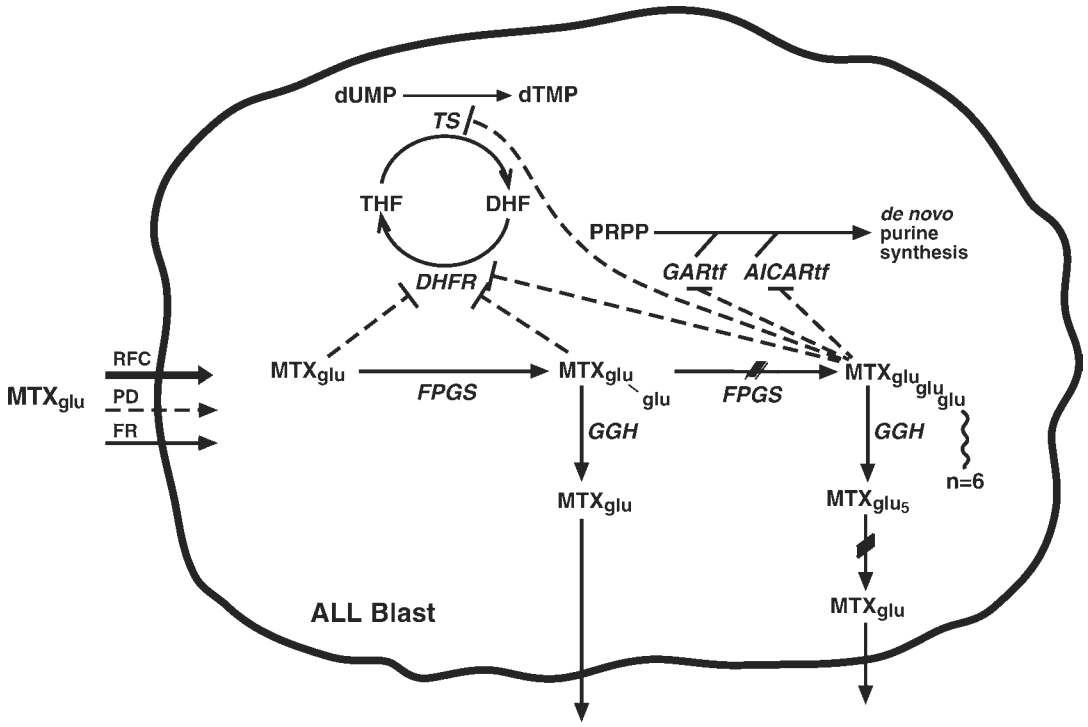

Fig. 1. Overview of methotrexate disposition and effects in leukemic lymphoblasts. MTXglu, methotrexate; $MTXglu_{n>1}$, methotrexate polyglutamates; RFC, reduced folate carrier; PD, passive diffusion; FR, folate receptor; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; GGH, γ-glutamyl hydrolase; TS, thymidylate synthetase; THF, tetrahydrofolate; DHF, dihydrofolate; PRPP, 5-phosphoribosyl pyrophosphate; GARtf, 5'-phosphoribosylglycinamide transformylase; AICARtf, aminoimidazole carboxamide ribonucleotide transformylase. (From ref. *173*.)

Thus, polyglutamation of methotrexate enhances cytotoxicity by multiple mechanisms, including longer retention in leukemic cells and greater inhibition of target enzymes *(24–26)*. It has also been shown that MTXPG accumulation is more avid in ALL blasts than in many normal tissues, providing a basis for selective cytotoxicity to leukemic cells and selective rescue of normal cells with leucovorin *(27,28)*. MTXPG are hydrolyzed by folylpolyglutamate hydrolase (FPGH), a lysosomal enzyme that cleaves glutamic acid residues from the polyglutamate form of methotrexate, to short-chain MTXPGs *(29)*. Some FPGH is also secreted from the lysosome into cytosol, but the enzyme is substantially less active at cytosolic pH (approx 7.4) than lysosomal pH (approx 4.5) *(30)*. To be cleaved by lysosomal FPGH, MTXPGs must enter the lysosome via a facilitative process that has not been fully characterized *(30)*. FPGH hydrolysis favors the cleavage of MTXPGs with increasing γ-glutamyl chain lengths *(30)*, and the enzyme can act as either an endopeptidase (cleaving interior glutamyl bonds) or an exopeptidase (cleaving the terminal glutamic acid residue). Eventually, free methotrexate is removed from the cell through a separate energy-dependent pathway that includes members of the multidrug-resistant protein transporter family *(31,32)*.

3. MECHANISMS OF METHOTREXATE RESISTANCE: RATIONALE FOR HIGH-DOSE METHOTREXATE

Methotrexate resistance may occur by a number of mechanisms: (1) RFC dysfunction; (2) DHFR amplification or mutation; (3) decreased FPGS activity; and/or (4) increased γ-glutamyl hydroxylase (GGH) activity *(26)*. Impaired RFC function or decreased RFC expression may decrease the amount of methotrexate that enters cells. Methotrexate transport has been shown to differ in the leukemic cells of patients with untreated vs relapsed ALL. Only 13% of untreated ALL patients were considered to have impaired methotrexate transport into leukemic cells, compared with 71% of relapsed patients ($p < 0.0001$). Two-thirds of samples (6 of 9) with impaired methotrexate transport exhibited a decrease in RFC expression *(33)*.

Resistance due to altered DHFR may result from increased expression of DHFR, amplification of the *DHFR* gene, and mutations leading to a decrease in binding of methotrexate to DHFR *(26,34–36)*. In experimental tumor systems, methotrexate resistance has been acquired through decreased transport and amplification of DHFR *(26,34,36)*. The genetic basis for methotrexate-resistant cell lines has been linked to *DHFR* gene amplification and point mutations, resulting in amino acid changes in the target enzyme, although the latter has yet to be observed in patients *(34,36,37)*. In one series, low-level (two- to fourfold) amplification of the *DHFR* gene was observed in approx 31% of relapsed childhood ALL patients after treatment that included low-dose methotrexate *(35)*.

Inefficient polyglutamation of methotrexate in leukemic cells results in less accumulation of $MTXPG_{4–6}$, which has been associated with decreased effectiveness of methotrexate therapy *(38)*. In vitro experiments have shown that increased FPGS activity is associated with greater methotrexate cytotox-

icity in CHO cells *(39)*. We observed higher FPGS activity in the leukemic blasts of patients with B-lineage ALL compared with T-lineage ALL and acute myeloid leukemia (AML), which resulted in greater long-chain MTXPG accumulation in patients with B-lineage ALL *(40)*. In this regard, both in vitro and in vivo studies have revealed lineage differences in FPGS activity and long-chain MTXPG accumulation. Galpin et al. *(41)* demonstrated a threefold increase in total and long-chain MTXPG accumulation in the NALM6 human B-lineage ALL cell line compared with the CEM T-lineage cell line, corresponding to higher FPGS activity in the NALM6 cells. In primary leukemic cells from patients, FPGS activity was greater in B-lineage ALL than T-lineage ALL blasts 44 h after methotrexate exposure (1249 vs 308 pmol/h/mg protein, $p = 0.03$), and FPGS activity increased to a greater extent in B-lineage lymphoblasts (188%) compared with T-lineage lymphoblasts (37%) after in vivo treatment with methotrexate ($p = 0.003$) *(40,41)*. Similarly, inefficient polyglutamation of methotrexate in T-lineage ALL was recently associated with lower FPGS activity and a lower FPGS-to-FPGH ratio in T-lineage vs B-lineage ALL *(42)*. Human CCRF-CEM T-lineage leukemia cells have been shown to develop acquired resistance to methotrexate by increasing FPGH activity, compared with the methotrexate-sensitive parental cell line *(43)*. Resistance to methotrexate secondary to high FPGH has not been established as a common mechanism in vivo; however, an elevated FPGH-to-FPGS ratio in ALL blasts has been associated with impaired methotrexate polyglutamation ($p < 0.05$) *(44)*.

Systemic clearance of HDMTX is highly variable among children with normal renal and hepatic function (>fivefold range), resulting in a corresponding range of systemic exposure if all patients are treated with the same dose of HDMTX. This raised the question of whether lower systemic exposure to methotrexate may influence EFS in children with ALL *(45,46)*. Thus, studies at St. Jude Children's Research Hospital (SJCRH) and the Pediatric Oncology Group (POG) found a greater risk of ALL relapse in children with B-lineage ALL who had more rapid systemic clearance of high-dose (1000 mg/m^2) methotrexate (Figs. 2 and 3) *(47–49)*. These findings led to the subsequent observation that children with lower exposure to methotrexate (steady-state plasma concentration <16 μ*M* during a 24-h infusion of HDMTX 1000 mg/m^2) had shorter durations of continuous complete remission (Fig. 4). More recently, Seidel et al. *(45)* also observed that children with ALL who have faster methotrexate clearances have a worse outcome.

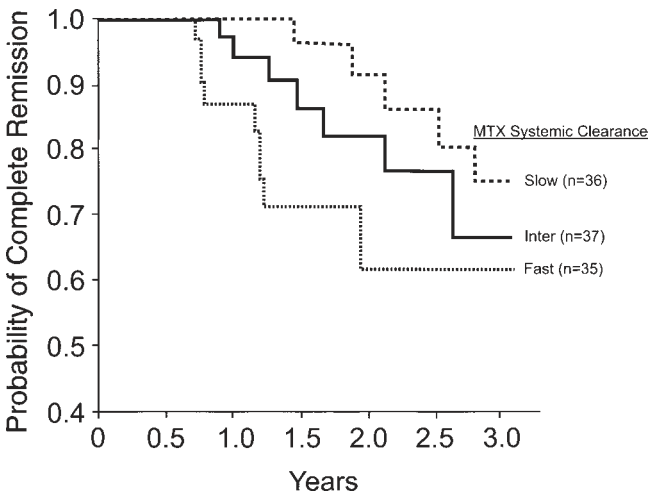

Fig. 2. Kaplan-Meier curves of complete remission durations according to the median rate of methotrexate (MTX) clearance in three groups of patients (slow, 44.7–71.9 mL/min/m^2, $n = 36$; intermediate, 72–83 mL/min/m^2, $n = 37$; and fast, 84–132 mL/min/m^2, $n = 35$). Statistical comparisons showed a significant difference between the fast- and slow-clearance groups ($p = 0.01$), but the differences between the fast- and intermediate-clearance ($p = 0.30$) and the slow- and intermediate-clearance ($p = 0.07$) groups were not significant. (From ref. *47*.)

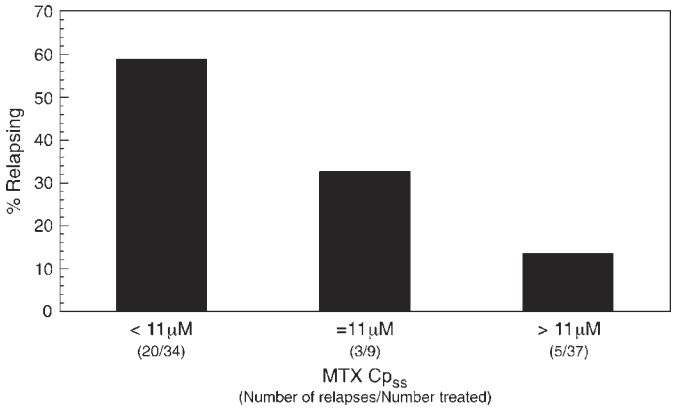

Fig. 3. Relation of systemic methotrexate clearance to leukemic relapse in children with higher risk non-T-, non-B-ALL treated on POG 8698 protocol, as described by Camitta et al. *(49)*. Patients were subdivided by whether their median steady-state methotrexate plasma concentrations (MTXCp_{ss}) were less than the population median (<11 μ*M*, $n = 34$), equal to the population median (11 μ*M*, $n = 9$), or greater than the population median (>11 μ*M*, $n = 37$). The bars represent percentages of patients relapsing in the three subgroups, after comparable durations of follow-up (approximately 4 yr). (From ref. *172*.)

4. CLINICAL PHARMACOKINETICS

4.1. Distribution

Following the administration of intravenous methotrexate, the steady-state volume of distribution ranges from 40 to 80% of body weight *(50–53)*. Methotrexate is only moderately bound to plasma protein (e.g., 50–60%) at plasma methotrexate concentrations >1 μ*M* *(50,54,55)*; thus, interpatient differences in protein binding are not a major source of variability in methotrexate disposition and effects.

Even though the cerebrospinal fluid (CSF) distribution of methotrexate is relatively low, serum methotrexate concentrations do influence CSF methotrexate concentrations. Intravenous methotrexate doses of 500 mg/m^2 over 24 h resulted in a mean CSF (0.6 μ*M*) to serum (20 μ*M*) concentration ratio of approx 3%, whereas a single 50-mg intravenous methotrexate dose produced a peak CSF to serum methotrexate ratio of 0.06% *(56)*. HDMTX (1000 mg/m^2 over 24 h) without intraventricular or intrathecal methotrexate administration produces a distribution ratio of lumbar CSF (0.27 μ*M*) to mean serum steady-state methotrexate concentration (15.8 μ*M*) of 2.3% *(57)*. The higher CSF methotrexate concentrations following HDMTX provide

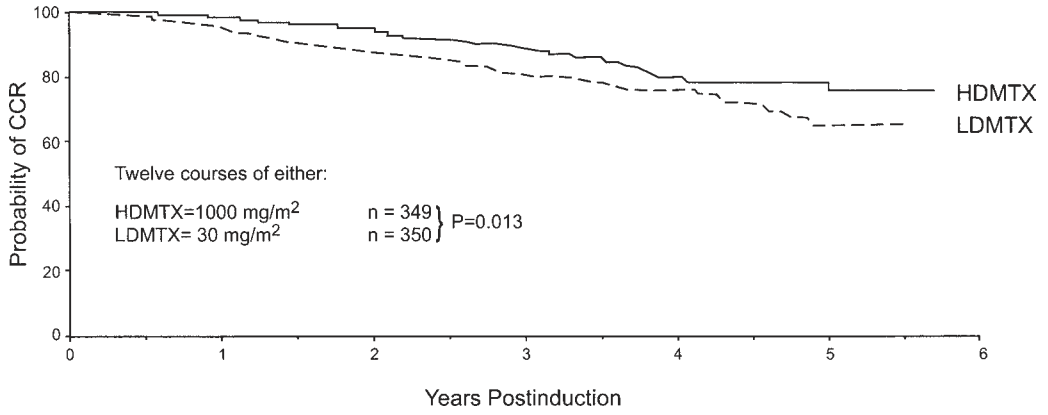

Fig. 4. Kaplan-Meier analysis of the continuous complete remission (CCR) durations for patients randomized to regimens containing either high-dose methotrexate (HDMTX) or low-dose (LD)MTX. Patients receiving the HDMTX regimen (n = 349) demonstrated a superior CCR rate compared with that achieved with the LDMTX regimen (80.3% vs 75.9%, p = 0.013). (From ref. *10*.)

an important rationale for this treatment. It should be noted that low-dose intrathecal methotrexate administration (6.25 mg/m^2) effluxes from the CSF and increases systemic methotrexate concentrations by approximately 0.1 μ*M* *(56)*.

HDMTX administration in patients with pleural effusions or ascites significantly alters methotrexate disposition by serving as a reservoir for methotrexate, often sustaining plasma methotrexate concentrations beyond the conventional duration of leucovorin rescue *(58,59)*. A decreased gastrointestinal transit rate secondary to complete or partial gastrointestinal obstruction, or drugs that substantially slow gastrointestinal motility (i.e., vincristine), can increase enterohepatic cycling of methotrexate and sustain methotrexate plasma concentrations following HDMTX *(60)*.

4.2. Catabolism

Methotrexate can be either activated by metabolism to MTXPG, as discussed above, or inactivated to either 2,4-diamino-N^{10}-methylpteroic acid (DAMPA) or 7-hydroxymethotrexate (7-OH-MTX). Metabolism of methotrexate by intestinal bacteria can result in the removal of the glutamate residue from the parent drug, forming 4-amino-4-deoxy-N^{10}-methylpteroic acid. The inactive metabolite DAMPA constitutes <5% of excreted drug in the urine from 0 to 24 h after HDMTX infusions and up to approx 25% of the excreted drug in the urine between 24 and 48 h after HDMTX infusions *(61,62)*. 7-OH-MTX, resulting from hydroxylation catalyzed by hepatic aldehyde oxidase, can constitute 20–46% of drug excreted in the urine when HDMTX is given as a 24-h infusion *(63)*. The percentage excreted as 7-OH-MTX is much lower following short (e.g., 4-h) methotrexate infusions *(64,65)*. Although it is a relatively weak inhibitor of DHFR compared with methotrexate *(66,67)*, 7-OH-MTX is a substrate for FPGS, and there is evidence that 7-OH-MTXPG can inhibit folate-dependent enzymes, AICAR transformylase, and thymidylate synthetase, but polyglutamation does not further enhance DHFR inhibition *(63)*. However, the levels of 7-OH-MTXPG in leukemic cells in vivo are apparently too low to contribute significantly to methotrexate's antileukemic effects. In vitro, 7-OH-MTX has been shown to compete with methotrexate for intracellular transformation to polyglutamate derivatives, but the clinical relevance of this observation remains unclear *(68)*.

4.3. Elimination

The major route of methotrexate elimination is renal excretion of unmetabolized drug. Human and animal studies have demonstrated that methotrexate renal clearance involves glomerular filtration, tubular secretion, and tubular reabsorption. Patients with impaired renal function (<60 mL/min/m^2) should not be treated with HDMTX, because they have markedly lower methotrexate clearance and because HDMTX can further reduce renal function *(69)*.

At methotrexate serum concentrations of up to 1000 μ*M*, renal clearance of the drug is much lower (<50 mL/min/m^2) than observed when serum concentrations are low (0.2–0.4 μ*M*). At these lower concentrations, methotrexate renal clearance is 6–49% greater than the glomerular filtration rate, consistent with active tubular secretion *(70,71)*. High methotrexate concentrations in the urine can exceed the solubility of methotrexate at a pH below 7.0, which is thought to be responsible for intrarenal precipitation of methotrexate and renal failure in the absence of appropriate hydration and alkalinization of the urine.

Biliary excretion represents <10% of overall methotrexate clearance *(50,53)*. Methotrexate undergoes uptake, metabolism, and storage in the liver, and the parent drug can be excreted into bile and reabsorbed into the systemic circulation from the small intestine. After lower dose intravenous methotrexate (<80 mg/m^2), 0.4–20% of the dose can be found in the biliary tract *(72–74)*.

5. CELLULAR PHARMACOKINETICS

5.1. Uptake of Methotrexate

5.1.1. Reduced Folate Carrier

The RFC functions as a bidirectional transporter of reduced folates and structurally unrelated anions. It has a higher affinity for reduced folates (0.7–1 μ*M*) than either methotrexate (5–6 μ*M*) or folic acid (100–200 μ*M*) *(75,76)*. The accumulation of methotrexate at plasma methotrexate concentrations

ranging from 1 to 20 μ*M* depends largely on the level of RFC expression and function *(19,20)*. In addition, nonspecific anions in sufficiently high concentrations can inhibit the interaction between folates and the RFC *(75,77,78)*.

5.1.2. Folate Receptor

The FR mechanism of cell entry contributes to methotrexate influx at very low extracellular concentrations of the drug (n*M*). This receptor has a 10–30-fold higher affinity for folic acid and reduced folates (n*M*) compared with methotrexate. FR-mediated methotrexate transport by potocytosis becomes maximal at extracellular drug concentrations of 0.05–0.1 μ*M* in receptor-positive cells *(79,80)*, resulting in maximal influx of methotrexate into cells when this mechanism predominates. It was therefore hypothesized that fractionated LDMTX would be as effective as HDMTX in leukemic cells that rely predominately on FR for methotrexate entry *(81)*. However, clinical studies have failed to support this hypothesis, demonstrating significantly lower intracellular methotrexate accumulation in ALL blasts of patients treated with LDMTX vs HDMTX *(21)*. This outcome probably reflects the low abundance of FRs on ALL blasts *(80)*, so that methotrexate enters leukemic lymphoblasts predominantly via the RFC, which saturates at much higher methotrexate concentrations.

5.1.3. Passive Diffusion

It is hypothesized that HDMTX maximizes RFC-mediated transport, allowing methotrexate to enter leukemic cells by passive diffusion. This concept has gained support from in vitro data suggesting that at higher extracellular concentrations (>20 μ*M*) methotrexate enters cells by RFC-mediated transport and passive diffusion *(20,82)*; however, the administration of HDMTX does not show a proportional increase in intracellular methotrexate concentration as the extracellular methotrexate concentration increases. Thus, the clinical advantage of methotrexate doses that produce concentrations above 50–100 μ*M* may relate more to achieving cytotoxic concentrations in sanctuary sites such as the central nervous system (CNS) and testes than to a proportional increase of methotrexate in leukemic cells in the bone marrow or other systemic sites.

5.2. Efflux of Methotrexate

5.2.1. Energy-Dependent Exporter

Methotrexate is typically removed from cells by a glucose- and ATP-dependent exporter that does not interact with the substrate until it has entered a cell *(83,84)*. To date, there has not been any definitive clinical evidence of methotrexate resistance associated with increased activity of this energy-dependent exporter, whose role in drug resistance appears negligible compared with that of other energy-dependent exporters, such as the multidrug-resistant proteins (MRPs) *(85)*.

5.2.2. Multidrug-Resistant Proteins

The MRP family of proteins belongs to a superfamily of ABC transporters and contains at least six distinct members. MRP1, the multidrug-resistant protein; MRP2 and MRP3, multispecific anion transporters; and MRP4–6 homologues with uncertain function, although MRP4 has been linked to resistance to some antiretroviral nucleosides *(86)*. MRPs may confer resistance by decreasing intracellular methotrexate concentrations, as overexpression of MRP1–3 has produced resistance to methotrexate in vitro *(31,87,88)*. Hooijberg et al. *(31)* demonstrated that overexpression of MRP1 and MRP2 is associated with resistance to short-term exposure to methotrexate (<4 h) but does not confer resistance to long-term treatment. The resistance produced by MRP3 resembles that seen with MRP1 and MRP2, but there is neither an increase in glutathione export nor a decrease in the intracellular level of glutathione *(87,88)*. The exact mechanism of MRP-associated resistance to methotrexate, as well as its clinical importance, is an area of active investigation.

6. HIGH-DOSE METHOTREXATE VS LOW-DOSE METHOTREXATE IN CLINICAL TRIALS

Even though methotrexate therapy has been extensively studied in childhood leukemia and HDMTX therapy has several potential pharmacologic advantages (e.g., capacity to overcome resistance due to decreased RFC function, to maximize MTXPG in cells, and to penetrate sanctuary sites such as the CSF and testes), there are few randomized studies comparing HDMTX with low-dose (LD)MTX therapy (Table 1). In a randomized study of repetitive LDMTX (30 mg/m^2 every 6 h for six doses) vs HDMTX (1000 mg/m^2 over 24 h) during intensification therapy for children with lower-risk ALL, Mahoney et al. *(10)* demonstrated that high doses of the drug produced a significantly better rate of continuous complete remission (CCR) than did low doses (80.3% vs 75.9%; Fig. 4, $p = 0.013$) and a significantly lower CNS relapse rate (2.3% vs 5.1%, $p = 0.034$). It should also be noted that the "low-dose" methotrexate regimen was actually a much higher dose (180 mg/m^2) than that used in conventional low-dose regimens (20–40 mg/m^2).

The FRALLE group showed that HDMTX is beneficial to intermediate-risk childhood ALL patients who received a four-drug induction with daunorubicin in FRALLE 93B, a sequential randomization trial. During induction, patients received either daunorubicin (40 mg/m^2 for two doses) or idarubicin (8 mg/m^2 for two doses). For consolidation, those patients were then randomized to receive either LDMTX (25 mg/m^2) or HDMTX (8000 mg/m^2) for four courses. In the daunorubicin arm, HDMTX produced a significantly higher 4-yr disease-free survival (DFS) rate compared with LDMTX for patients who received daunorubicin during induction, at 90.9% vs 79.6% ($p = 0.04$). However, no difference in the 4-yr DFS rate was observed between HDMTX and LDMTX patients who received idarubicin during induction, at 85.9% vs 82.0% ($p = 0.53$). Additional analysis of relapsed patients showed that none of 30 patients with *TEL-AML1* treated with daunorubicin and HDMTX had a relapse event, whereas 2 of 22 patients with *TEL-AML1* treated with daunorubicin and LDMTX had a relapse event, at 100% vs 71.4% ($p = 0.038$) *(18)*.

In standard-risk ALL patients on Dana-Farber Cancer Institute (DFCI) protocol 81-01, Neimeyer et al. *(89)* observed a trend toward a better 7-yr EFS rate with HDMTX (4000 mg/m^2 over 1 h) compared with LDMTX (40 mg/m^2) during induction therapy, 82% vs 69% ($p = 0.13$), which continues after 10 years, at 82% vs 64% ($p = 0.10$) *(15)*. Failure to achieve statistical significance may have been caused in part to the randomization of only 77 patients and the design of this investigational win-

Table 1
Comparison of High-Dose Methotrexate Versus Low-Dose Methotrexate in Clinical Trials

Protocol	*Risk group/ sample size*	*Treatment phase with methotrexate*	*HDMTX dose/infusion duration*	*LDMTX dose/route*	*Treatment outcome*		*p value*
					HDMTX	*LDMTX*	
POG 9005 *(10)*	Lower-risk HDMTX, 349 LDMTX, 350	Intensification	1000 mg/m^2 iv over 24 h (× 12 courses)	30 mg/m^2 po every 6 h × 6 (× 12 courses)	CCR (4-yr), 80.3%	CCR (4-yr), 75.9%	0.013
DFCI 81-01 *(15,89)*	Standard-risk HDMTX, 38 LDMTX, 39	Investigational window prior to remission induction	33000 mg/m^2 iv over 24 h × (1 dose) (n = 9) 4000 mg/m^2 iv over 24 h (× 1 dose) (n = 29)	40 mg/m^2 po (× 1 dose)	EFS (7-yr), 82% EFS (10-yr), 82%	EFS (7-yr), 69% EFS (10-yr), 64%	0.13 0.10
FRALLE 93B *(18)*	Intermediate-risk DNR arm HDMTX, 157 LDMTX, 142 IDR arm HDMTX, 159 LDMTX, 148	Consolidation	8000 mg/m^2 iv over 24 h (× 4 courses)	40 mg/m^2 po (× 4 courses)	DNR arm DFS (4-yr), 90.9% IDR arm DFS (4-yr), 85.9%	DNR arm DFS (4-yr), 79.6% IDR arm DFS (4-yr), 82.0%	0.04 0.53

Abbreviations: HDMTX, high-dose methotrexate; LDMTX, low-dose methotrexate; iv, intravenous; po, oral; CCR, complete continuous remission; EFS, event-free survival; DFS, disease-free survival; DNR, daunorubicin; IDR; idarubicin; POG, Pediatric Oncology Group; DFCI, Dana-Farber Cancer Institute.

dow study, which was not to determine EFS. Subsequently, in response to the trend toward improved EFS in patients randomized to HDMTX, DFCI 87-01 randomized patients to receive only one dose of either HDMTX (4000 mg/m^2 over 24 h) or LDMTX (40 mg/m^2) during remission induction. However, no difference was observed in the 5-yr EFS rate for patients randomized to either HDMTX or LDMTX, at 77% vs 73%, p = 0.48 *(15)*. The overall 5-yr EFS rate was 78% for standard-risk patients in this study, which was lower than expected, presumably due to an increased CNS relapse rate in male patients. The decreased EFS rate may be attributed to the addition of HDMTX or LDMTX without intensive intrathecal therapy and the discontinuation of cranial irradiation. The 5-yr EFS rate increased in standard-risk patients to 87% in the subsequent trial, DFCI 91-01, in which all patients received HDMTX (4000 mg/m^2 over 24 h) during remission induction, and cranial irradiation (1800 cgy) was reinstituted in standard-risk male patients *(15)*.

In addition, Lange et al. *(90)* observed that intermediate-dose methotrexate (IDMTX) (500 mg/m^2 over 24 h) given during consolidation and maintenance to a smaller population of intermediate-risk patients (n = 164) did not show an advantage in terms of relapse rates or EFS compared with LDMTX. These results are not informative in comparing the outcomes of IDMTX and LDMTX since IDMTX patients received one-third less vincristine (1.5 mg/m^2) and prednisone (40 mg/m^2) pulses during maintenance, compared with LDMTX patients. In addition, the methotrexate dose in this study (500 mg/m^2) was too low to produce concentrations comparable to those associated with a favorable outcome *(48)*.

Although IDMTX (500 mg/m^2 over 24 h) has not produced an improvement in EFS in other studies, it has been shown to influence the pattern of relapses. For example, the Cancer and Leukemia Group B (CALGB) 7611 trial compared IDMTX (500 mg/m^2 over 24 h) plus intrathecal methotrexate with standard therapy and cranial irradiation plus intrathecal methotrexate during intensification in standard- and intermediate-risk ALL patients. Even though the 12-yr CCR rate was 40% for both treatment regimens *(91)*, standard-risk patients receiving IDMTX plus intrathecal methotrexate had fewer bone marrow relapses (9 of 117 vs 24 of 120, $p < 0.01$), but more CNS relapses (23 of 117 vs 8 of 120, $p = 0.01$) compared with patients receiving standard therapy and cranial irradiation plus intrathecal methotrexate *(92)*. The intermediate-risk patients receiving IDMTX plus intrathecal methotrexate also had more CNS relapses (31 of 142 vs 10 of 127; $p = 0.03$) than did the comparison group *(92)*. Thus, lower doses of IDMTX (500 mg/m^2) decrease systemic relapses, but do not provide adequate prevention of CNS relapses. However, more intensive intrathecal therapy or the use of higher doses of methotrexate can overcome the problem of inadequate CNS therapy with IDMTX.

Various studies have demonstrated that conventional and more intensive ALL chemotherapy regimens utilizing HDMTX (1000–8000 mg/m^2) produce excellent EFS rates in childhood ALL (Table 2) *(1,2,4–6,8,9,13,15–18,89,93–100)*. The EFS rates in low- and intermediate-risk childhood leukemia patients have improved to >75% *(101)*. Because almost all ALL protocols utilize 6–10 antileukemic agents, it is difficult to determine the importance of any single medication or the optimal dosage of any one agent. Although excellent results can be achieved with ALL regimens that include little or no HDMTX (Table 2) *(11)*, this outcome requires treatment intensification with agents that often invoke more serious toxicities than those induced by methotrexate (e.g., steroids, avascular necrosis or bone fractures; anthracyclines, cardiotoxicity; epipodophyllotoxins, second malignancies; and L-asparaginase, thrombosis or pancreatitis).

The effectiveness of HDMTX in consolidation therapy has been further documented in studies of the Berlin–Frankfurt–Münster (BFM) Group *(1,2,16)*. In BFM 86 and 90, for example, the methotrexate dose was increased from 500 mg/m^2 over 24 h, as in BFM 81 and BFM 83, to 5000 mg/m^2 over 24 h. There was also an addition of vincristine and triple intrathecal therapy (TIT) consisting of methotrexate, cytarabine, and prednisolone. Cytarabine and teniposide doses were increased and fractionated, and cyclophosphamide was partially replaced by ifosfamide. Overall, BFM 86 and 90 demonstrated an improved 8-yr EFS rate by comparison with BFM 83 (75.9 and 70.4% vs 62.3%) *(16)*. The Dutch Childhood Leukemia Study Group (DCLSG) ALL-7 study, designed to be identical to BFM 86, produced a 5-yr EFS rate of 65.3%, which was significantly lower than the 73% achieved in BFM 86 (p = 0.02); however, when the analysis was based on similar patient populations, there was no significant difference in 5-yr EFS rates (64.6% vs 67%) *(6)*.

Although HDMTX is most commonly used as part of consolidation or continuation therapy for childhood ALL, it has also been used during "window therapy" prior to conventional remission induction and during remission induction. In the DFCI 81-01 study, there was a trend toward improved treatment outcome among children randomized to receive a single dose of HDMTX (4000 mg/m^2) compared with those who received LDMTX (40 mg/m^2) as "upfront window" therapy prior to conventional remission induction therapy; however, the small patient number (n = 77) probably contributed to the lack of statistical significance (p = 0.10) *(15)*.

Since 1991, HDMTX (1000 mg/m^2 over 24 h) has been given as "window therapy" prior to conventional remission induction as a component of Total Therapy protocols at SJCRH. In the XIIIA study, upfront HDMTX was well tolerated, with a <10% incidence of grade 3 or 4 mucositis *(38)*. Furthermore, the use of upfront HDMTX at 1000 mg/m^2 did not compromise the delivery of remission induction therapy, as the number of days required to complete induction plus consolidation therapy was similar among patients treated in different arms of Total Therapy XIIIB (mercaptopurine alone or combined with LDMTX or HDMTX, or no upfront window therapy, $p = 0.379$) or among the 49 children who were randomized to receive low, medium, or high steady-state concentrations of HDMTX on the Total Therapy XIV protocol (p = 0.35). The 5-yr EFS rate for patients treated in Total Therapy studies XIIIA and XIIIB was 81%, but it is too early to assess differences in outcome in subgroups of patients who were randomized to upfront HDMTX vs LDMTX *(8,101)*.

The use of intensive systemic chemotherapy (with or without HDMTX) and CNS preventive therapy has also increased

Table 2
Summary of Methotrexate Dosages and Outcome in Various ALL Treatment Protocols

Protocol	*Treatment phase with methotrexate*	*HDMTX*	*MTX dose (mg/m^2)*	*Duration of infusion (h)*	*Treatment outcome*
CCG-105 *(3)*	Interim maintenance and maintenance	No	15, then 20	po	IR, 73% EFS (5-yr)
UKALL X *(106)*	Maintenance	No	20	po	All risk, 60% EFS (10-yr)
CCG (standard vs augmented therapy) *(11)*	Interim maintenance and maintenance	No	15 or 100, then 20	Not stated	HR, 75% EFS (5-yr)
DFCI-85-01, 87-01, and 91-01 *(170)*	Intensification	Yes	130	1	Infant, 54% EFS(5.6-yr)
CALGB 7611 *(92)*	Intensification	Yes	500	24	SR, 67% and IR, 49% CCR (4-yr)
BFM 83 *(1,105,171)*	Continuation	Yes	500	24	All risk, 62% EFS (10-yr)
POG 9005 *(10)*	Intensification	Yes	1000	24	LR, 85% EFS (4-yr)
POG 9006 *(94)*	Intensification	Yes	1000	24	HR, 61% EFS (4-yr)
POG 8399 *(12)*	Intensification	Yes	1000	24	LR, 94% EFS (4-yr)
POG 8602 *(7)*	Consolidation	Yes	1000	24	SR, 78% EFS (5-yr)
SJCRH Total XIII *(8)*	Remission induction (~1/3 pts.)	Yes	1000	24	LR and HR, 81% EFS (5-yr)
	Consolidation and continuation	Yes	2000	24	
SJCRH Total X *(124)*	Postremission	Yes	1000	24	SR, 67% CCR (4-yr)
SJCRH Total XII *(9)*	Continuation	Yes	1500 (Conv) vs 1500–3000 (Cp_{ss} = 20–30 μM; Ind)	24	Conv, 66% EFS (5-yr) Ind, 76% EFS (5-yr)
SJCRH Total XI *(4,95)*	Consolidation	Yes	2000 (2 doses)	24	LR, 80% and HR, 67% EFS (5-yr)
DCLSG ALL 6 *(5,14)*	Consolidation	Yes	2000	24	NHR, 81% EFS (10-yr)
AIEOP 91 *(96,97)*	Consolidation	Yes	SR = 2000	24	All risk, 69% EFS (8-yr)
			IR = 5000	24	
DFCI 81-01 *(89)*	Remission induction	Yes	33,000 (9 pts.)	24	SR and HR, 67% EFS (7-yr)
			4000	1	SR and HR, 86% EFS (7-yr)
DFCI 91-01 *(15,98)*	Additional intensification	Yes	4000	1	VHR, 72% EFS (7-yr)
AIEOP 88 *(97)*	Consolidation	Yes	5000	24	All risk, 65% EFS (10-yr)
DCLSG ALL 7 *(6,14)*	Consolidation	Yes	5000	24	All risk, 76% EFS (10-yr)
EORTC 58881 *(17,99)*	Interval therapy	Yes	5000	24	SR, 73% EFS (8-yr)
BFM 86 *(1,16)*	Consolidation	Yes	5000	24	All risk, 69% EFS (10-yr)
BFM 90 *(2,16)*	Consolidation	Yes	5000	24	All risk, 76% EFS (8-yr)
NOPHO ALL 92 *(13)*	Consolidation and maintenance	Yes	5000	24	All risk, 78% EFS (5-yr)
UKALL XI *(93,100)*	CNS-directed	Yes	6000 or 8000	24	All risk, 60% EFS (8-yr)
FRALLE 93B *(18)*	Consolidation	Yes	8000	24	DNR arm: SR, 91% DFS (4-yr) IDR arm: SR, 86% DFS (4-yr)

Abbreviations: HDMTX, high-dose methotrexate; MTX, methotrexate; DFS, disease-free survival; DNR, daunorubicin; EFS, event-free survival; CCR, complete continuous remission; Conv, conventional therapy; IDR, idarubicin; Ind, individualized therapy; MT, maintenance therapy; LR, lower risk; SR, standard-risk; NHR, non-high-risk; IR, intermediate-risk; HR, high-risk; VHR, very-high-risk; CNS, central nervous system; CCG, Children's Cancer Group; DFCI, Dana-Farber Cancer Institute; CALGB, Cancer and Leukemia Group B; BFM, Berlin-Frankfurt Münster Group; POG, Pediatric Oncology Group; SJCRH, St. Jude Children's Research Hospital; DCLSG, Dutch Childhood Leukemia Study Group; AIEOP, Italian Association of Pediatric Hematology and Oncology; EORTC, European Organization for Research and Treatment of Cancer; NOPHO, Nordic Society of Pediatric Hematology and Oncology.

the EFS rate in higher risk patients, including those with T-cell ALL, from roughly 50% to >65% *(1,102–104)*. In the BFM 86 study, T-cell ALL patients had a favorable EFS rate after receiving this intensive treatment regimen, similar to results obtained at the DFCI with comparable therapy, 73% vs 70% *(104)*. In fact, the improved outcome for T-lineage ALL in BFM 86 was attributed in part to the use of HDMTX at 5000 mg/m^2, compared with 500 mg/m^2 in BFM 83, with the EFS rate for T-ALL increasing from 53% (10-yr) in BFM 83 to 71% (10-yr) in BFM 86 *(1,15,105)*. The SJCRH Total Therapy XI protocol yielded a 5-yr EFS rate of 67% in patients with "higher risk" ALL (including T-lineage ALL), who received a multiagent chemotherapy regimen that included 2 doses of HDMTX (2000 mg/m^2) during consolidation therapy *(4,95)*.

An advantage of using HDMTX, rather than other leukemic agents, in systemic therapy may be related to the avoidance of the serious adverse effects that can occur in long-term ALL survivors (e.g., late cardiac failure and second malignancies). The DCLSG achieved a 10-yr EFS rate of 81.5% in non-high-risk ALL patients, treated without anthracyclines, alkylating agents, and cranial irradiation to minimize the long-term complications that may occur with intensive leukemic therapy *(14)*. A low CNS relapse rate (1.1%) was obtained with HDMTX (2000 mg/m^2 over 24 h for three doses) plus TIT in the first year of maintenance therapy, with dexamethasone used for induction and pulses during maintenance treatment *(14)*. Overall, these HDMTX regimens produced outcomes comparable to those of chemotherapy regimens that included anthracyclines, alkylating agents, and epipodophyllotoxins in the consolidation phase of therapy *(1,7,94,106)*.

The efficacy of therapy that includes HDMTX in the induction, continuation, and consolidation phases has been established clinically. However, there are only a few well-designed studies directly comparing HDMTX with LDMTX. The results of three such trials *(10,18,89)* indicate that HDMTX therapy improves outcome, which is consistent with in vitro and human in vivo *(21,27,38)* studies showing higher MTXPG levels in ALL blasts after HDMTX compared with LDMTX. Likewise, HDMTX achieves higher methotrexate concentrations in the CSF than does LDMTX *(1,107,108)*.

6.1. Clinical Pharmacokinetics Versus Outcome

The SJCRH Total Therapy X protocol prospectively randomized 108 children with standard-risk ALL to receive 15 doses of HDMTX (1000 mg/m^2 over 24 h) as part of postremission therapy. Leucovorin rescue consisted of 30 mg/m^2 intravenously at 36 and 42 h followed by 3 mg/m^2 orally at 54, 66, and 78 h (total, 69 mg/m^2) after the start of the HDMTX infusion. Methotrexate clearance was not related to the duration of therapy or total number of doses administered. Interpatient differences in methotrexate clearance resulted in large variability in the steady-state methotrexate serum concentration (range of mean steady-state methotrexate serum concentration among patients, 9.3–25.4 μ*M*). Patients whose median steady-state methotrexate serum concentrations were <16 μ*M* (*n* = 59) were 3.2 times more likely to have any type of relapse (hematologic, CNS, or testes) and 6.9 times more likely to have a hematologic relapse

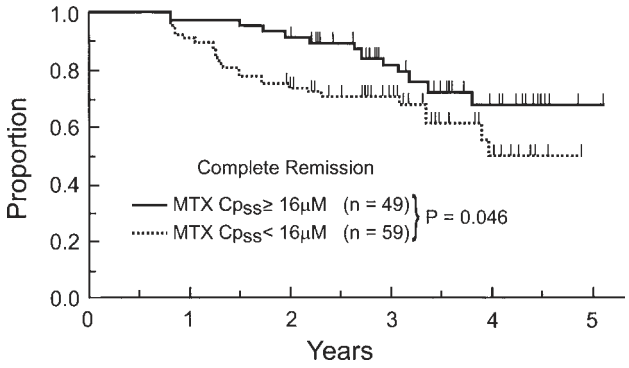

Fig. 5. Kaplan-Meier curves of complete remission durations for patients with median steady-state concentrations of methotrexate (MTX Cp_{ss}) <16 μ*M* or ≥16 μ*M*. The difference between the two curves is significant ($p = 0.046$ by the Breslow test). (From ref. *48*).

at 3.5 yr, compared with patients with median steady-state methotrexate serum concentrations of ≥16 μ*M* (*n* = 49; Fig. 5). These data indicate that the level of systemic exposure to methotrexate can significantly influence the probability of relapse in children with standard-risk ALL *(48)*.

The Pediatric Oncology Group (POG) study 8399 evaluated HDMTX (1000 mg/m^2 over 24 h) given every other week during 24 wk of intensification therapy, following remission induction. Fifty-eight of the 59 lower-risk children with B-precursor ALL received all 12 doses of HDMTX with leucovorin rescue (15 mg/m^2 every 6 h for nine doses). The mean steady-state methotrexate serum concentration was 11 μ*M*. At 4 yr, the EFS rate was 94%, with only three patients experiencing a hematologic relapse (one had a concurrent CNS relapse) *(12)*. The EFS rate decreased to 82.4% at 7 yr *(49)*. In these lower-risk patients, there was no relation between methotrexate systemic exposure and EFS. Interestingly, when the Total Therapy X results were subsequently reanalyzed to determine whether methotrexate exposure was related to outcome in "good-risk" patients, as classified by the POG, there was no significant relationship, similar to the POG 8399 findings. However, for "higher-risk" patients, methotrexate clearance was significantly related to the risk of any relapse on therapy and to hematologic relapse over the entire follow-up of 7.3 yr *(47,48,109)*. These findings indicate that the level of systemic exposure to methotrexate is more important in patients with an increased risk of relapse and may be less important in patients whose disease is highly sensitive to combination chemotherapy.

The favorable results in children with lower-risk B-precursor ALL in the POG 8399 study led to evaluation of children with higher-risk B-precursor ALL in POG 8698. Newly diagnosed higher-risk B-precursor ALL patients (*n* = 80) received HDMTX (1000 mg/m^2) administered over 24 h (biweekly) in a 24-wk intensification therapy following induction therapy similar to that in POG 8399. However, the leucovorin rescue was reduced to 5 mg/m^2 (intravenously or orally) every 6 h for five doses (total, 25 mg/m^2) starting 48 h after the start of the HDMTX infusion. The median steady-state methotrexate serum concentration during the HDMTX infusions was 11 μ*M*. The 4-yr EFS rate, 57.4%, was significantly related to

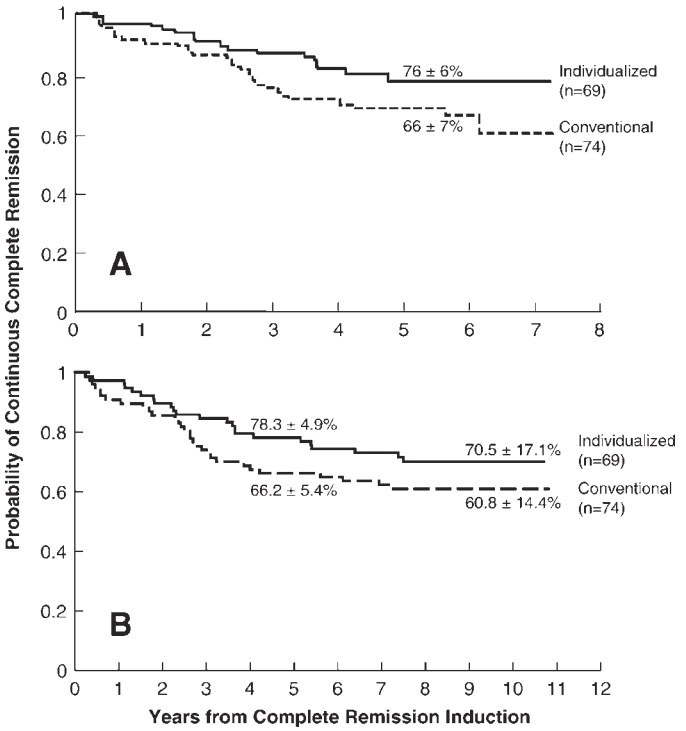

Fig. 6. Kaplan-Meier plots of continuous complete remission (CCR) durations in patients with B-lineage ALL. **(A)** The CCR estimates through 7 yr of follow-up were significantly higher in the 69 patients receiving individualized treatment compared with the 74 patients receiving conventional treatment ($p = 0.02$). **(B)** With follow-up extending to 10 yr, the curve for individualized therapy remains higher than the one for conventional therapy, although the level of statistical significance has diminished ($p = 0.171$). Values on the curves are mean probabilities (± SE) at 5, 5.5, and 10 yr of follow-up. (A: From ref. *9*.)

the level of systemic exposure to methotrexate. Relapse occurred in 59% of patients (20 of 34) with a median steady-state methotrexate serum concentration less than the population median (11 μ*M*), compared with 33% of patients (3 of 9) with a steady-state methotrexate serum concentration equal to the median of 11 μ*M* (Fig. 3), and in only 14% of patients (5 of 37) with median steady-state methotrexate serum concentrations >11 μ*M* *(49)*.

These findings thus corroborate the earlier St. Jude data indicating a relation between relative systemic methotrexate exposure and remission rates in childhood ALL. It is not surprising that the precise methotrexate concentration with prognostic importance differed in the St. Jude and POG studies (16 vs 11 μ*M*), because other treatment variables (e.g., leucovorin dose, hydration, and other therapy) can shift the critical value. In this regard, the leucovorin dose was 69 mg/m^2 in the SJCRH protocol vs 25 mg/m^2 in the POG 8698 protocol; thus it is not surprising that the critical methotrexate exposure level in the SJCRH protocol was higher (16 vs 11 μ*M*).

Subsequent to the above findings, a prospective randomized trial was conducted to assess the clinical benefit of individualizing the dose of HDMTX and other ALL pulse chemotherapy, to avoid low systemic exposure in patients with fast clearance *(9)*. All newly diagnosed ALL patients randomized to individualized doses of HDMTX (adjusted to maintain a steady-state methotrexate concentration >20 μ*M* and ≤30 μ*M*) had significantly fewer courses of treatment with systemic exposures below the target range compared with patients randomized to conventional HDMTX (fixed 1500 mg/m^2 over 24 h) during postinduction therapy ($p < 0.001$) *(9)*. More importantly, B-lineage ALL patients treated with individualized therapy had a significantly better CCR rate ($p = 0.02$) compared with that achieved with conventional dosing (76% vs 66% 5-yr CCR; Fig. 6A). By contrast, no statistically significant difference was observed in relapse risk between individualized and conventional HDMTX therapy for patients with T-lineage ALL *(9)*. We postulate that the lack of benefit from individualized therapy in T-lineage ALL may be related to having used the same target plasma concentration for both B-lineage and T-lineage ALL, given the significant lineage differences in MTXPG accumulation we have subsequently observed *(21)*. With longer follow-up (approximately 10 yr), the CCR curve for individualized therapy remains above the conventional therapy treatment, although the small number of patients has diminished the level of statistical significance ($p = 0.171$; Fig. 6B).

6.2. Methotrexate Polyglutamate Accumulation

Whitehead et al. *(27)* suggested that decreased MTXPG formation in ALL blasts may predict a worse clinical outcome, because children with B-lineage ALL had a better 5-yr EFS rate (65% vs 12%) if their leukemic blasts accumulated higher concentrations of MTXPGs (>500 pmol/10^9 lymphoblasts) after in vitro incubation with 1 μ*M* methotrexate ($p = 0.01$) *(110)*. In vitro studies with human ALL cell lines have illustrated the importance of both methotrexate concentration and duration of exposure in maximizing MTXPG accumulation *(41,111,112)*. For example, the accumulation of MTXPGs was greater in B-lineage (NALM6) than in T-lineage (CEM) cells after exposure to 1 μ*M* of methotrexate for 24 h compared with 5 μ*M* for 4 h, even though the total AUCs for extracellular exposure were similar *(41)*. It has long been recognized that both the concentration and the time of exposure are important determinants of methotrexate cytotoxicity *(107,113–115)*.

To determine whether methotrexate dose plays an important role in MTXPG accumulation in vivo, Synold et al. *(21)* measured MTXPG concentrations in leukemic cells from patients randomized to single-agent treatment with HDMTX vs LDMTX. At 44 h after methotrexate administration, significantly higher concentrations of $MTXPG_{4-6}$ were achieved in the ALL blasts of patients treated with HDMTX (1000 mg/m^2) compared with children treated with LDMTX (986 vs 355 pmol/10^9 lymphoblasts, $p = 0.0001$) *(21)*. There were also significant lineage differences in MTXPG accumulation in vivo (Fig. 7). Eighty-four percent of the patients with B-lineage ALL receiving HDMTX exceeded the minimum intracellular MTXPG concentration (>500 pmol/10^9 blasts) identified by Whitehead et al. *(27)* for a more favorable clinical outcome, compared with only 57% of B-lineage ALL patients receiving LDMTX *(21)*. Furthermore, none of the patients with T-lineage ALL had an MTXPG concentration >500 pmol/10^9 blasts when treated with

LDMTX, whereas three of four exceeded this value when treated with HDMTX. In addition, of 34 non-hyperdiploid B-lineage ALL patients who received HDMTX, 28 (82%) had an MTXPG level >500 pmol/10^9 blasts, compared with only 17 (53%) of 32 who received LDMTX.

Subsequently, Masson et al. *(38)* observed that greater lymphoblast accumulation of MTXPGs was associated with greater acute antileukemic effects in children with newly diagnosed B-lineage ALL. Patients who had complete clearing of circulating lymphoblasts within 4 d of single-agent treatment had significantly higher blast cell MTXPG concentrations at 44 h (2793 vs 602 pmol/10^9 blasts, $p = 0.0039$). This relationship is illustrated by the change in circulating blast counts observed after methotrexate treatment in individual patients (Fig. 8). Moreover, the estimated $MTXPG_{4-7}$ concentration needed to inhibit *de novo* purine synthesis by 95% (>483 pmol/10^9 blasts) was more likely to be achieved in vivo by administration of HDMTX rather than LDMTX (81% vs 46%, $p < 0.0001$) *(38)*.

6.3. Duration of Methotrexate Infusion

In addition to uncertainty about the optimal dosage of methotrexate for childhood ALL, there is also uncertainty about the optimal duration of infusion for HDMTX administration. Methotrexate infusions ranging from 5 min to 36 h have been used in clinical protocols, with no systematic assessment of the optimal duration. It is not clear whether peak plasma concentration, steady-state plasma concentration, or the duration of methotrexate exposure is more important for optimizing the antileukemic effects of methotrexate.

The length of exposure to methotrexate has been most rigorously assessed in preclinical in vitro and in vivo models. It has been shown that longer exposures to equal extracellular concentrations result in greater methotrexate polyglutamate formation and cytotoxicity *(26,116–120)*. Preclinical data showing equivalent bone marrow suppression at an AUC of 120,000 n*M*·h (12-h exposure) *(121)* and at 24,000 n*M*·h (24-h exposure) suggest that duration of exposure is an important determinant of methotrexate effect, as long as a minimum threshold extracellular methotrexate concentration is exceeded *(26,117–122)*. Unfortunately, there are no adequate clinical trials comparing the duration of infusion between HDMTX regimens, although the proposed POG 9904 and 9905 studies will compare 4-h vs 24-h HDMTX infusions, albeit at different doses (2000 mg/m^2 vs 1000 mg/m^2). This question will be addressed in the new SJCRH Total Therapy XV protocol, by measuring MTXPG levels in ALL blasts after 4-h vs 24-h infusions of methotrexate (1000 mg/m^2).

It is important to recognize that the optimal infusion length of methotrexate may differ for children with different lineage and genetic subtypes of ALL. Clinical data indicate that the level of systemic exposure following 1000–1500 mg/m^2 of methotrexate over 24 h significantly affects the EFS of children with B-lineage but not T-lineage ALL *(9,48)*. Total Therapy XIIIA demonstrated that, given the same dosages of methotrexate, the total accumulations of MTXPGs and long-chain $MTXPG_{4-6}$ were significantly lower in T-lineage compared with B-lineage blasts (Fig. 7) *(21)*. The underlying mechanisms for these lineage and ploidy differences have only been partly elucidated; thus, the optimal strategy for overcoming them remains to be established. Moreover, it is not known whether infusion length is a more important determinant of MTXPG accumulation in hyperdiploid B-lineage, nonhyperdiploid B-lineage, or T-lineage ALL. These questions will also be addressed in the Total Therapy XV protocol.

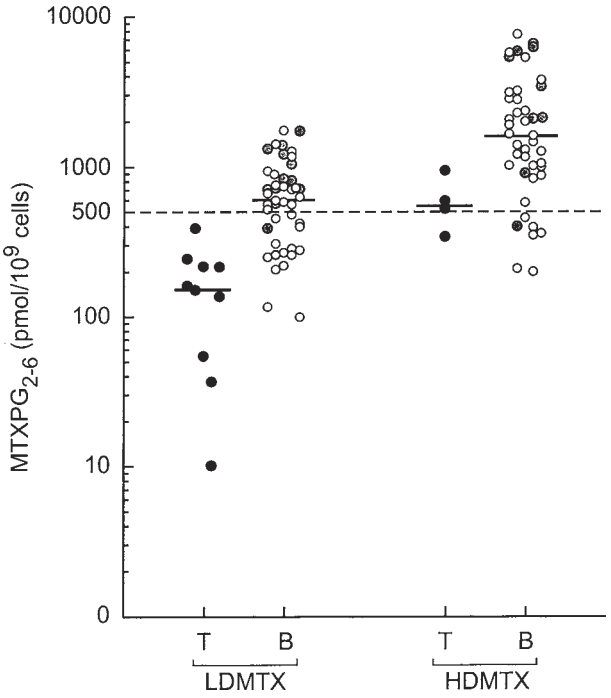

Fig. 7. Intracellular methotrexate polyglutamates (MTXPG) concentrations in ALL blasts following exposure to either low-dose methotrexate (LDMTX) or high-dose (HD)MTX in children with newly diagnosed ALL. Each symbol depicts an individual patient: ●, B-lineage hyperdiploid (>50 chromosomes; $n = 21$); ○, B-lineage non-hyperdiploid (≤50 chromosomes; $n = 66$); and ●, T-lineage hyperdiploid and non-hyperdiploid ALL ($n = 14$). Solid lines depict median values for each group. The dashed line represents 500 pmol/10^9 blasts, the minimum intracellular MTXPG concentration (following in vitro incubation with MTX) that was identified by Whitehead et al. *(27)* as being associated with a more favorable outcome in children with B-lineage ALL. HDMTX achieved MTXPG concentrations above 500 pmol/10^9 cells in a significantly higher proportion of patients with B-lineage ($p = 0.001$) or T-lineage ($p = 0.01$) ALL. Non-hyperdiploid B-lineage blasts demonstrated more $MTXPG_{2-6}$ formation than did hyperdiploid B-lineage blasts after HDMTX: median 3371 vs 1332 pmol/10^9 cells ($p = 0.037$). (Modified from ref. *21*.)

Prolonged HDMTX infusions may afford a mechanism to overcome intrinsic resistance to methotrexate. An increased duration of exposure (24 h) to methotrexate has been shown in vitro to circumvent methotrexate resistance due to overexpression of MRP1, MRP2, and MRP3, which cause resistance to short (4-h) exposures to methotrexate *(31)*. However, the impact of MRP expression in vivo remains unknown for childhood ALL.

6.4. Central Nervous System Prophylaxis and Treatment

The efficacy and intensity of systemic therapy, particularly the dose of methotrexate, can significantly affect the

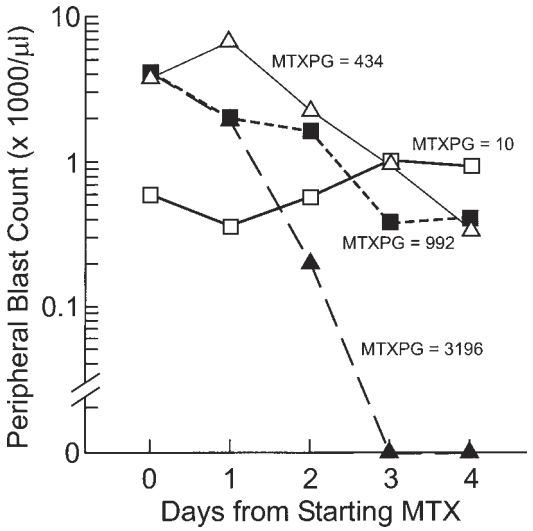

Fig. 8. Examples of four patterns of circulating blast counts observed in individual patients after methotrexate treatment, before the start of remission induction chemotherapy. Open symbols depict two patients treated with LDMTX: one who had the lowest methotrexate polyglutamate (MTXPG) concentration (□) and one with MTXPG concentrations comparable to the median value for the LDMTX group (△). Closed symbols depict two patients treated with HDMTX: one who had MTXPG concentrations comparable to the median for the HDMTX group (■), and one with MTXPG concentrations greater than twofold higher than the median for the HDMTX group (▲). (From ref. *38*.)

treatment of CNS leukemia. In a randomized study of childhood ALL, oral repetitive LDMTX (30 mg/m^2 every 6 h for six doses) with intrathecal methotrexate for early intensification was inferior CNS therapy compared with HDMTX (1000 mg/m^2 over 24 h) *(10)*. In addition, Mahoney et al. *(123)* demonstrated higher-than-expected bone marrow and CNS relapse rates with repetitive LDMTX (30 mg/m^2 every 6 h for five doses) when it was given with intravenous 6-mercaptopurine in lower-risk B-lineage ALL patients. These data nicely illustrate that intrathecal methotrexate with repetitive LDMTX does not provide adequate CNS prophylaxis and that HDMTX is more effective for eradicating CNS leukemia.

Several investigators have found that the frequency of isolated CNS relapse following CNS preventive therapy with HDMTX (1000–5000 mg/m^2) and intrathecal methotrexate is similar to cranial irradiation (1800 cgy) and sequential chemotherapy *(124–127)*. The SJCRH Total Therapy X Study *(124)* demonstrated a better CCR rate ($p = 0.049$) and better overall survival rate (>90%; $p < 0.0001$) among intermediate-risk ALL patients randomized to therapy including HDMTX (1000 mg/m^2 over 24 h) administered during the postremission period, compared with cranial irradiation and sequential chemotherapy containing epipodophyllotoxins (Fig. 9). Therefore, low-risk and intermediate-risk ALL patients are able to receive intensified intrathecal methotrexate and maintain an isolated CNS relapse rate of <2% without cranial irradiation.

It is important to note that HDMTX without intrathecal treatment does not provide adequate prevention of CNS relapse. Pullen et al. *(128)* observed that extended triple intrathecal therapy without HDMTX resulted in isolated CNS relapse rates of 2.8 and 7.7% in POG good-risk and poor-risk patients, compared with 9.6 and 12.7% in comparable subgroups given HDMTX (1000 mg/m^2) pulse therapy without intrathecal therapy every 8 wk. These data indicate that HDMTX pulse therapy without extended triple intrathecal therapy does not provide adequate protection against CNS relapse.

6.5. Methotrexate-Induced Neurotoxicity

Intrathecal, low-dose, and high-dose administration of methotrexate can all lead to neurotoxicity, but the precise mechanism for this effect remains unknown *(129–136)*. HDMTX (500–8000 mg/m^2) is generally well tolerated in patients with ALL *(137)*, although the frequency of seizures associated with such therapy is reported to be approx 2% *(47)*.

Systemic methotrexate therapy and lower doses of HDMTX (500–2000 mg/m^2), have been associated with acute and chronic encephalopathy and seizures in childhood ALL. Kubo et al. *(138)* reported seizures and hemiparesis 5 d after a single course of HDMTX (1000 mg/m^2). Other investigators have described the development of hemiparesis with excessive dysphagia or transient leukoencephalopathy within 5–13 days after HDMTX (500–1800 mg/m^2) *(139,140)*. Permanent leukoencephalopathy with transient hemiparesis has been reported following administration of intravenous cytarabine (1000 mg/m^2) and methotrexate (1000 mg/m^2) *(141)*. The incidence of leukoencephalopathy has been decreased by not administering HDMTX after cranial irradiation. Overall, severe encephalopathy following HDMTX (<5000 mg/m^2) is quite rare in childhood leukemia patients *(142–144)*.

Methotrexate-mediated neurotoxicity may also occur after simultaneous administration of systemic and intrathecal methotrexate. Rubnitz et al. *(143)*. reported that 8 of 259 ALL patients developed transient neurologic changes within 13 d following simultaneous administration of HDMTX (2000 mg/m^2) and TIT. These neurologic changes resolved within 3 d. Importantly, seven of the eight patients were able to receive additional HDMTX courses without further complications.

HDMTX is an alternative to cranial irradiation for CNS prophylaxis in children with ALL; it reduces growth retardation *(145,146)* and secondary CNS malignancies *(147)* but results in long-term neuropsychological consequences. In a prospective study by Ochs et al. *(148)* comparing neuropsychological functioning in patients with a median time of 6 yr post remission, children receiving HDMTX (1000 mg/m^2) with intrathecal methotrexate or 1800 cgy cranial radiation with intrathecal methotrexate had comparable decreases in neuropsychological function. Statistically significant decreases were observed in overall and verbal intelligence quotients and in arithmetic achievement for both groups. However, standard tests of intelligence and academic achievement were not significantly different from baseline scores.

Even though LDMTX has been shown to produce only mild toxicity, its use does not eliminate the risk of neurotoxicity. For example, Winick et al. *(149)* reported acute neurotoxicity in 25 of 113 patients treated with fractionated LDMTX (25 mg/m^2 every 6 h for four doses) and TIT. Despite removing cytarabine

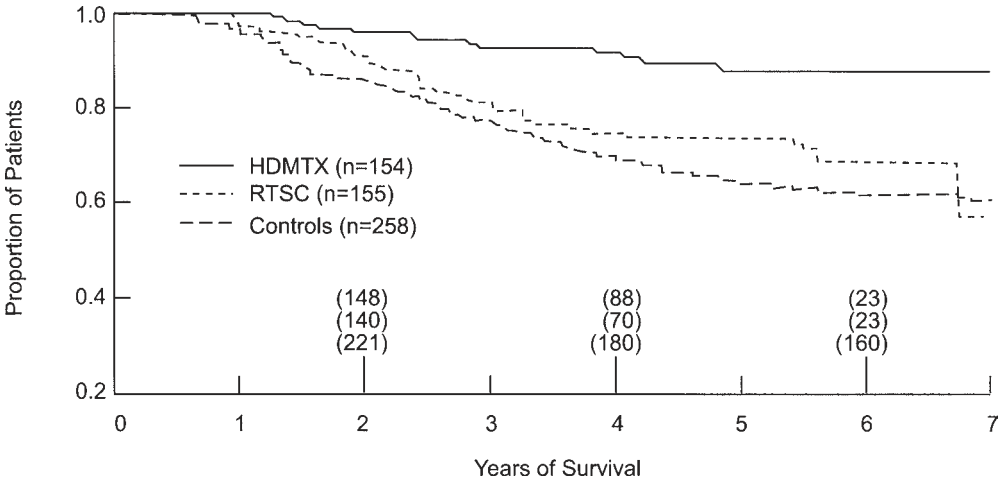

Fig. 9. Overall survival by treatment group: (1) high-dose methotrexate (HDMTX) 1000 mg/m^2 over 24 h once a week for 3 wk plus intrathecal methotrexate for CNS prophylaxis followed by maintenance chemotherapy consisting of HDMTX 1000 mg/m^2 over 24 h every 6 wk for 18 mo plus intrathecal methotrexate in addition to weekly oral maintenance 6-mercaptopurine (50 mg/m^2) and methotrexate (25 mg/m^2); (2) RTSC, cranial irradiation and sequential chemotherapy (six drugs in rotating pairs); and (3) Controls, patients from SJCRH Total Therapy VIII and IX studies. Total Therapy X HDMTX and RTSC patients were newly diagnosed standard-risk ALL with an initial leukocyte count $<100 \times 10^9$ cells/L, no mediastinal mass, no blast cells in cerebrospinal fluid, and negative blast cells for sheep erythrocyte rosette formation and surface immunoglobulin; Total Therapy VIII and IX control patients have similar clinical features. The differences in survival are highly significant ($p < 0.0001$). Numbers in parentheses denote patients who reached or exceeded the corresponding times from diagnosis. (From ref. *124*.)

from the TIT, the high frequency of seizures persisted. In the 22 patients with methotrexate-induced neurotoxicity, most of the neurotoxic events manifested as seizures, but transient neurologic deficits were also observed. Of note, addition of leucovorin (5 mg/m^2 given 24 and 36 h after the last dose of methotrexate) eliminated seizures in 24 subsequently evaluated patients.

6.6. Other Toxicities

The use of HDMTX with leucovorin rescue in the 1970s was associated with a mortality rate of approx 6% *(150)*, primarily in patients who had delayed clearance of methotrexate, thereby sustaining cytotoxic methotrexate plasma concentrations beyond the period of leucovorin rescue. Severe myelosuppression with sepsis or hemorrhage and renal failure were the major reasons for HDMTX-related morbidity and mortality *(150)*. With appropriate attention to hydration, urinary alkalinization, renal function, and monitoring of methotrexate serum concentrations, fatal toxicity has essentially been eliminated *(60,151–153)*. Today, toxicities associated with methotrexate therapy are largely limited to reversible mucositis, myelosuppression, hepatic and renal dysfunction, and neurotoxicity *(10,148,154,155)*. As is the case with its antileukemic effects, methotrexate toxicity is determined by the length of time that cytotoxic concentrations are maintained without adequate leucovorin rescue *(156,157)*. The increased risk for toxicity is associated with methotrexate plasma concentrations >1 μ*M* at approx 42 h after the start of therapy *(60)*, unless sufficient leucovorin is given *(152)*.

6.7. Prevention of Toxicity

Clinical pharmacokinetic monitoring and supportive care have improved the safety of HDMTX *(150)*, such that severe toxicity is now extremely rare *(154,158,159)*. Leucovorin (5-formyltetrahydrofolate), administered after HDMTX to prevent methotrexate-induced toxicity, is thought to rescue normal vs leukemic cells selectively because MTXPG formation is greater in leukemic cells than normal bone marrow and gastrointestinal cells, and it is more difficult to reverse methotrexate effects with leucovorin when MTXPG concentrations are high *(160–162)*. Leucovorin can compete with methotrexate transport into the cell, but, more importantly, it is metabolized to tetrahydrofolate, thereby replenishing reduced folate pools in cells and reversing the major cytotoxic effects of methotrexate. It is important to administer the minimum dose of leucovorin needed to rescue host cells, to avoid decreasing efficacy by "overrescue" *(163–165)*. Unfortunately, the lowest effective leucovorin dose must be empirically determined for each protocol and within the context of HDMTX therapy, as there are no precise pharmacologic or biochemical criteria to guide dose selection.

The clinical efficacy of leucovorin "rescue" relates to the timing and dose of leucovorin and methotrexate *(166)*. Leucovorin is usually administered within 24–48 h following the start of methotrexate therapy. If effective leucovorin rescue is not begun within 48 h, the cytotoxic effects of methotrexate may be irreversible *(156,167–169)*. Leucovorin doses used with HDMTX protocols range from 5 mg/m^2 to approx 300 mg/m^2, depending on the dose of HDMTX and the context of its administration. Leucovorin doses are usually given every 6 h for three to six doses, unless there are high methotrexate serum concentrations (e.g., >0.5 μ*M*) 48 h after the start of methotrexate administration, necessitating more prolonged leucovorin administration. Therefore, an appropriate balance between the total dose of methotrexate and the amount of leucovorin rescue must be empirically achieved to minimize toxicity and maximize efficacy.

7. CONCLUSIONS

There is little argument that methotrexate is an active antileukemic agent that has played an important role in increasing EFS rates in childhood ALL. However, the optimal dose, duration of infusion, and treatment context for HDMTX remain to be precisely elucidated. Most investigators contend that HDMTX is superior to conventional LDMTX, although some clinicians remain unconvinced. There is unequivocal evidence that HDMTX achieves higher concentrations of active MTXPGs in ALL blast patients *(21)*. Furthermore, some clinical trials have demonstrated an inferior CCR rate with LDMTX vs HDMTX, and historical comparisons show a better outcome with newer protocols that contain higher doses of methotrexate *(1,2,104)*. The advantages of HDMTX include its ability to achieve higher MTXPG levels in leukemic blasts and to reach higher concentrations in "sanctuary sites" such as the CNS and testes.

Because of wide interpatient pharmacokinetic variability, lineage and ploidy differences in MTXPG accumulation in ALL blasts, and the paucity of studies that directly compare different HDMTX doses or HDMTX to LDMTX, it is not yet possible to determine precisely the optimal dose of methotrexate for each subtype of childhood ALL. It is our view that methotrexate doses of 1000–1500 mg/m^2 may be adequate for hyperdiploid B-lineage ALL, 2000–2500 mg/m^2 for non-hyperdiploid B-lineage ALL, and approximately 5000 mg/m^2 for T-lineage ALL. However, better designed studies are needed to answer some of the following questions definitively: what are the ALL subtypes and treatment protocols in which HDMTX is more efficacious than LDMTX; what are the optimal doses of HDMTX for specific lineage and genetic subtypes of ALL; what is the optimal duration of HDMTX infusion; and what is the optimal dose and duration of leucovorin rescue? Such information should lead to even more effective use of this active agent in curative therapy for childhood ALL.

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26 Role of Methotrexate in the Treatment of Acute Lymphoblastic Leukemia

BRUCE M. CAMITTA AND BARTON A. KAMEN

CONTENTS

1. INTRODUCTION

In 1941, a small amount of the new vitamin folic acid was concentrated from approximately 4 tons of spinach *(1)*. Within 5 yr this compound had been synthesized *(2)*. Because folic acid was known to cure some patients with megaloblastic anemia, it was hypothesized that a deficiency of the vitamin might also play a role in the pathogenesis of acute lymphoblastic leukemia (ALL). However, when they were treated with folic acid, children with ALL appeared to have an acceleration of their disease. Based on this observation, the reverse was hypothesized: a compound that interfered with folic acid metabolism might be useful for the treatment of ALL. In 1948, aminopterin (a 4-NH_2 analog of folic acid) was reported by Farber et al. *(3)* to decrease blast counts in children with that disease. Because of its superior therapeutic index, methotrexate (MTX) rapidly replaced aminopterin for clinical use.

In the five decades since Farber's seminal publication, MTX has emerged as the preeminent folate antagonist for the treatment of ALL and other malignancies. However, despite a wealth of new information regarding normal folic acid metabolism, methotrexate pharmacology, and the pathogenesis of ALL, there are still fundamental gaps in our knowledge of these factors. Thus, to some extent, the use of MTX for the treatment of ALL remains empiric and controversial. In this chapter we review some of the issues that are currently unresolved.

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2. FOLIC ACID AND METHOTREXATE METABOLISM

The normal nonstressed individual requires 2–3 mg/kg/d of folic acid. Most cells take up folate by a high-capacity, low-affinity reduced folate carrier (encoded on chromosome 21). Intracellular folate retention is favored by polyglutamation [the addition of one to six additional glutamate residues by folylpolyglutamyl synthase, (FPGS), encoded on chromosome 9]. The polyglutamates are better substrates than folic acid for folate-requiring enzymes. A separate high-affinity, low-capacity folate receptor (encoded on chromosome 11) helps to conserve folic acid in specific tissues (proximal renal tubules, choroid plexus, and syncytiotrophoblasts). As an organic acid, the multiple organic ion transport system (MOATS), located on the proximal renal tubule and bile canalicular membranes, may also secrete folates.

Reduced folates are involved in many metabolic pathways (Fig. 1). These include the synthesis of purines and pyrimidines, conversion of homocysteine to methionine, and provision of methyl groups for metabolism of multiple substrates. Therefore, it is not surprising that a deficiency of folic acid may result in illnesses as diverse as megaloblastic anemia, cardiovascular disease, cognitive disorders, failure of neural tube closure, and an increased risk of developing cancer.

As a folic acid analog, MTX utilizes the same mechanisms for absorption and metabolism. It may inhibit folate-requiring enzymes directly or by inducing folate deficiency [by competing with folate for absorption or via inhibition of dihydrofolate reductase (DHFR)]. Polyglutamation increases the potency of

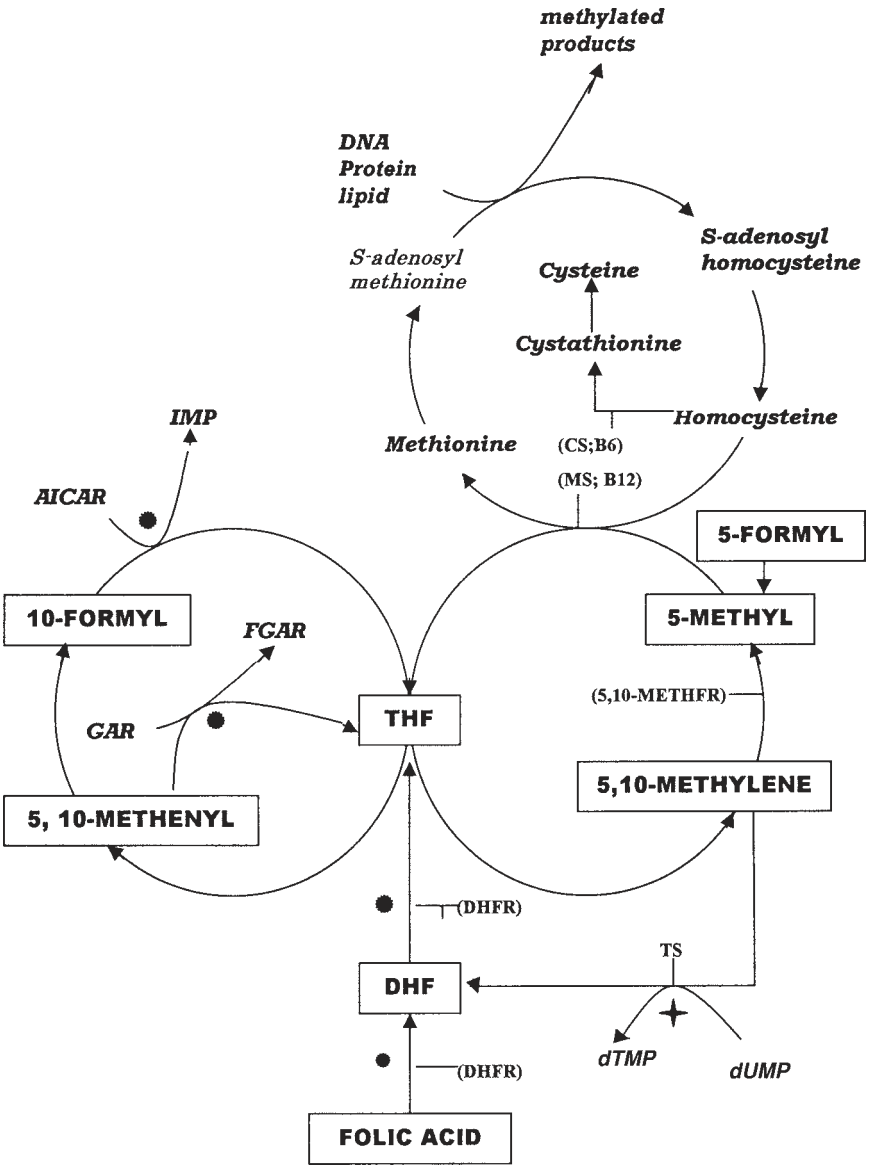

Fig 1. Folic acid metabolism. Different forms of folic acid are indicated in boxes. (●) , reactions inhibited by MTX or MTX polyglutamates. Thymidylate synthetase (✦) is inhibited primarily by MTX polyglutamates. Homocysteine can also be converted to methionine by betaine: homocysteine methyl transferase using trimethyl glycine as a cofactor. 5-formyl, leucovorin; AICAR, 8-amino-4-imidazole carboxamide ribonucleotide; B6, pyridoxal phosphate; B12, methylcobalamine; CS, cystathionine synthetase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; FGAR, formyl-GAR; GAR, glycinamide ribonucleotide; IMP, inosine monophosphate; 5,10-METHFR, 5,10-methylene tetrahydrofolate reductase; MS, methionine synthetase; TS, thymidylate synthetase; dUMP, deoxyuridine monophosphate.

MTX as an inhibitor of folate-requiring enzymes. Therefore, it is logical to envision that the relative concentrations of MTX and reduced folates and their affinities for specific enzymes determine the effects of MTX in malignant and nonmalignant cells. In this regard it is pertinent to point out that the normal plasma folate concentration ranges from 5 to 15 ng/mL (about 50 mg total serum folate in an adult). Therefore, even low doses of MTX (30 mg; 30,000 μg) "flood" the plasma compartment, in comparison with amounts of reduced folates. Finally, the efficacy of MTX may also be affected by factors that determine a cell's susceptibility to apoptosis.

3. GENERAL TREATMENT STRATEGY FOR CHILDHOOD ALL

Event-free survival rates for children with ALL improved from 5% in the mid-1960s to 75% by 1999. Key components of this remarkable progress include (1) three- or four-drug induction therapy (producing remissions in 98–99% of patients); (2) central nervous system (CNS) "prophylaxis" (decreasing the incidence of CNS relapse to <10%); (3) an intensive phase of treatment (designed to decrease the numbers of residual lymphoblasts rapidly, thereby decreasing the likelihood of muta-

tion to drug resistance or treating already resistant cells); and (4) a continuation phase of treatment.

MTX currently plays no role in induction treatment. There is little controversy regarding its use during continuation therapy, but there is considerable difference of opinion regarding its optimal role during intensification and CNS prophylaxis. In the following sections we review some of these issues.

4. SYSTEMIC TREATMENT

Given the above considerations, we previously postulated that once the extracellular concentration of MTX exceeds the K_m for the reduced folate transport system, increasing the concentration further would not increase MTX uptake or cytotoxicity. In patients treated with oral, intramuscular, or intravenous MTX, there was only a three-fold difference in red blood cell MTX content despite 25-fold differences in cumulative MTX doses *(4,5)*. In addition, there was no difference in red blood cell MTX levels between patients who received oral MTX in divided doses (25 mg/m^2 every 6 h × 4 every other wk) and patients who received intravenous MTX (1000 mg/m^2 iv alternating weekly with 20 mg/m^2 im). Because red blood cells accumulate MTX only during the nucleated stages of their development, red cell MTX was thought to be a reasonable surrogate for the uptake of MTX by leukemic cells. However, measurement of MTX uptake by lymphoblasts in patients with newly diagnosed ALL has shown that treatment with high-dose MTX (1.0 g/m^2 iv in 24 h) produced three-fold higher MTX levels in leukemic blasts than did treatment with low-dose MTX (30 mg/m^2 orally every 6 h for six doses) *(6)*. For each MTX dose, the accumulation of MTX in B-lineage leukemic blasts was threefold greater than in T-lineage blasts. The higher levels of intracellular MTX correlated with a greater suppression of purine synthesis and a greater decrease in circulating blasts *(7)*.

In another study, when lymphoblasts from untreated patients were incubated with MTX in vitro, there was increased uptake and polyglutamation of MTX in cells from patients with a better prognosis *(8)*. A more recent study suggests that a higher ratio of MTX/folate uptake by leukemic cells is more predictive of favorable outcome than is MTX uptake alone *(9)*. It remains to be shown whether increased uptake of MTX by leukemic cells is directly responsible for a better response to therapy. An alternative hypothesis is that increased MTX uptake is merely a surrogate for a more important determinant of response such as susceptibility to apoptosis *(10)* or altered drug metabolism. Finally, even if increased uptake of MTX is important for therapeutic success, this does not mean that more is always beneficial. For example, at a concentration of 50 μM, MTX inhibits its own polyglutamation *(11)*. This drug level is easily achieved in vivo by a 24-hr infusion of 5 g/m^2.

What is the optimal schedule of MTX for treatment of ALL? In an early study, MTX was more effective and less toxic when given intramuscularly at 30 mg/m^2 twice weekly than when given at 3 mg/m^2/d orally *(12)*. In the L1210 system, delayed administration of leucovorin allowed the use of higher doses of MTX and improved survival *(13)*. In children, repeated intermediate-dose (500–1000 mg/m^2) MTX with delayed leucovorin rescue improved event-free survival in several studies *(14–17)*. This primarily reflected decreases in bone marrow and testicular relapses. There was little if any change in the rate of CNS relapse. In another study, repeated moderate-dose MTX (100–300 mg/m^2) was a key component of a regimen that improved the outcomes of patients with a poor prognosis (because of slow response to initial treatment) *(18)*. Children with T-lineage ALL appeared to benefit from addition of high-dose MTX (5 g/m^2 × 4) to a regimen of the Berlin–Frankfurt–Münster group (BFM) *(19)*. Finally, a single preinduction treatment with high-dose (4 or 33 g/m^2) MTX improved event-free survival in comparison with standard-dose (40 mg/m^2) MTX in a small randomized trial *(20)*.

Further support for using intermediate- or higher-dose MTX comes from pharmacokinetic and pharmacodynamic studies. Higher steady-state plasma MTX levels correlated with improved event-free survival in several studies *(21,22)*. This benefit was seen primarily in patients at higher risk of relapse. In an elegant follow-up study, the St. Jude team randomized patients to intensification with fixed doses of intermediate dose MTX, cytarabine, and etoposide or to the same intensification with doses adjusted to achieve "optimal" plasma drug levels *(23)*. The latter patients had a small but significant improvement in event-free survival (66% vs 76%; $p = 0.021$). The difference was primarily due to differences in time-dependent systemic exposure to methotrexate. However, although elimination of interpatient differences in pharmacokinetics and pharmacodynamics was beneficial, this study did not prove that higher doses of MTX are necessary. In addition, higher doses of MTX might be harmful in individual patients *(11)*.

Other studies have failed to demonstrate a therapeutic advantage for higher-dose MTX. The Pediatric Oncology Group (POG) compared two methods of intensification for children with standard-risk ALL *(24)*. One group received intermediate-dose MTX (1 g/m^2), whereas the other received divided dose oral methotrexate (30 mg/m^2 every 6 h × 6). MTX treatments were repeated every 2 wk for 12 doses. Patients in both groups received identical leucovorin rescue (5 mg/m^2 every 6 h × 5) and 6-mercaptopurine. There was a marginally significant advantage for the intermediate-dose MTX regimen (event-free survival 80.3% vs 75.9%; $p = 0.013$). However, this study was potentially flawed by standardization of the leucovorin dosing *(25)*. That is, the ratio of MTX:leucovorin was 40:1 in the intermediate-dose regimen but only 7.2:1 in the divided dose oral regimen. Additional evidence for "over-rescue" was an increased rate of CNS relapse in the oral regimen (7% vs 4%). Even 10 mg of leucovorin is sufficient to triple the cerebrospinal fluid folate level *(26)*.

In the UK ALLXI trial, patients with white blood counts $<50 \times 10^9$/L were randomized to receive three courses of high-dose MTX (6–8 g/m^2). Patients who received this treatment had a slightly lower risk of CNS relapse (9% vs 13%; $p = 0.007$), but there was no decrease in bone marrow relapse or event-free survival (69% vs 67%) *(27)*. In the FRALLE-93 study, intermediate-risk patients were randomized to low-dose (25 mg/m^2 × 7) or high-dose (8 g/m^2 × 4, plus low dose × 3) MTX during consolidation. The disease-free survival (83% vs 87%) and CNS relapse rates were comparable in both groups *(28)*. Finally, in the Children's Cancer Group (CCG) 139 study, low-dose (20 mg/m^2 weekly) oral MTX was com-

pared with low-dose oral MTX plus moderate-dose (500 mg/m^2 × 3 during consolidation; every 6 wk during continuation) intravenous MTX therapy for children with intermediate-risk ALL *(29)*. Event-free survival rates were equivalent (58% vs 57%) despite five- to six-fold differences in exposure to MTX.

The Dallas–Fort Worth consortium has been using divided-dose oral MTX (25 mg/m^2 every 6 h × 4) with limited leucovorin rescue (5 mg/m^2 24 and 36 h after the last dose of MTX) *(30)*. Almost 400 patients have been treated (results updated by B.A. Kamen and N.J. Winick, personal communication). Event-free survival is 81% at 5 yr. In the first cohort (244 patients), there were more patients with CNS relapse or secondary acute myeloid leukemia (AML) than bone marrow failures.

Despite these encouraging results, several caveats are in order. First, the earliest cohort received additional consolidation with etoposide and cytarabine. These medications were discontinued because of the high incidence of secondary AML. There has been no increase in the rate of relapse of patients who did not receive these agents (although follow-up has been shorter in the second cohort). Second, different regimens of intrathecal CNS prophylaxis were used. Although leucovorin decreased CNS toxicity, the use of intrathecal MTX alone vs triple intrathecal (TIT) prophylaxis did not affect the CNS relapse rate *(31)*. Third, L-asparaginase was given to most patients 2–4 h after divided-dose MTX. The effect of adding this drug is not known. Fourth, the study was uncontrolled. Finally, the divided-dose schedule was continued for nearly a year, much longer than in most protocols relying on higher-dose MTX for intensification. In contrast to the synchronously growing L1210 murine leukemia, childhood ALL is a nonsynchronous disease with a smaller growth fraction. In the latter setting, the time of exposure to MTX and the number of MTX courses may be very important for the effectiveness of treatment.

The apparent discrepancies in the results of the above studies might reflect differences in a number of factors, including (1) the amounts, timing, or numbers of MTX doses; (2) the amount and timing of leucovorin rescue; (3) concomitant chemotherapy; (4) the subtypes of leukemia; and (5) patient characteristics. Only direct, appropriately controlled comparisons of low-dose, divided-dose, and intermediate- or higher-dose MTX (with appropriate leucovorin rescue) will enable us to judge the relative efficacy, toxicities, and costs of these approaches. Convincing evidence that higher-dose MTX is necessary for optimal treatment of childhood ALL is currently not available.

5. CENTRAL NERVOUS SYSTEM PROPHYLAXIS

Cranial irradiation plus intrathecal MTX has been the standard prophylaxis for CNS leukemia for many years. Because of associated neurologic toxicities, alternatives to the use of irradiation have been sought. At high doses, the CSF:plasma ratio of MTX is only 1 or 2% *(32)*. Very high-dose systemic MTX (33 g/m^2) will decrease the number of cerebrospinal fluid (CSF) blasts in patients with CNS leukemia. It was equivalent to cranial irradiation plus intrathecal MTX for the prevention of CNS relapse in patients with intermediate-risk or higher-risk ALL *(33)*. However, at doses used in most protocols (0.5–2.0 g/m^2), there is little evidence that intermediate-dose MTX can substitute adequately for intrathecal medication (with or without irradiation) as CNS prophylaxis. The use of higher doses of systemic MTX might be more effective but also could result in greater CNS toxicities *(30)*.

Repeated doses of intrathecal MTX alone provide comparable CNS prophylaxis compared with cranial irradiation plus intrathecal MTX. This has been demonstrated for patients at lower *(34)*, intermediate *(35,36)*, and higher *(37)* risks of relapse. Important cautions regarding these data are that they are based heavily on patients with B-precursor ALL who received BFM-like systemic chemotherapy. Intrathecal MTX alone may be inadequate with nonintensive systemic chemotherapy regimens *(35)*. In addition, as discussed above, over-rescue with leucovorin might increase the risk of CNS relapse in patients treated with MTX-based regimens. The efficacy of intrathecal MTX alone in the setting of other types of chemotherapy must still be demonstrated.

The POG has used TIT medication (methotrexate, hydrocortisone, and cytarabine) for CNS prophylaxis for 25 yr. Although protection of the CNS appears similar to that seen with other regimens *(24)*, there have been no randomized comparisons of the three-drug regimen with intrathecal MTX alone. In this regard it is important to note that MTX and cytarabine may have synergistic, additive, or antagonistic effects when used on different schedules and in different cell lines in vitro *(38)*. Direct comparisons of the efficacy, toxicities, and costs of these approaches are needed.

6. NEUROLOGIC TOXICITY

A potential advantage of MTX-based treatment of ALL is a decrease in the acute and long-term toxicities that may be associated with anthracyclines, epipodophyllotoxins, and alkylating agents. However, recent data have associated MTX use with an increased incidence of neurotoxicities *(32,39)*. Potential pathogenic factors include (1) higher dose MTX; (2) intrathecal MTX; (3) TIT medication; (4) intrathecal treatment techniques (source and concentration of medications, volume of fluid removed, position during and after the spinal tap); (5) the dose and timing of leucovorin; (6) other systemic medications; (7) patient characteristics; and (8) interactions among the above. Review of the POG experience suggests that neurotoxicity is greatest when higher-dose MTX and intrathecal MTX are given at separate times during intensification. In that setting, intrathecal MTX is unaccompanied by leucovorin rescue. The data are inconclusive in regard to a possible role for TIT chemotherapy *(39)*. Similar conclusions may be drawn from the experience of the Dallas–Fort Worth group, who reported significant neurotoxicities in 10 of 72 patients treated with divided-dose MTX plus IT methotrexate: 6 of 42 who received divided-dose MTX plus intrathecal MTX and none of 24 treated with divided-dose MTX plus intrathecal MTX plus low-dose leucovorin *(31)*.

Methotrexate neurotoxicity may be placed into three broad categories: acute, subacute, and delayed. The reader is cautioned that there may be considerable overlap in clinical findings and pathogenic mechanisms among these groups.

Nausea, emesis, headache, lethargy, somnolence, and seizures characterize acute neurotoxicity, occurring within hours to days of MTX administration. These symptoms may be sec-

ondary to an increase of adenosine in the nervous system (due to inhibition by MTX of the conversion of 8-amino-4-imidzaole carboxamide ribonucleotide (AICAR) to inosine monophosphate with secondary inhibition of adenosine catabolism; Fig 1). Adenosine accumulation owing to inhibition of adenosine deaminase by deoxycoformycin resulted in similar symptoms *(40)*. Adenosine receptor antagonists (theophylline or caffeine) may lessen some of the above symptoms *(41)*.

Subacute neurotoxicity, occurring days to weeks after MTX administration, may be characterized by seizures, paresis, changes in affect, aphasia, dysesthesias, and other problems. The biochemical mechanisms may be complex but appear to be related to effects on folate homeostasis. Folate deficiency inhibits the conversion of homocysteine to methionine (Fig 1). Increased plasma and CSF homocysteine may damage endothelial cells *(42,43)*, possibly requiring adenosine (via *S*-adenosylhomocysteine) as a cofactor. Homocysteine and its metabolites also may cause neurotoxicity by a second mechanism: direct stimulation of *N*-methyl-D-aspartate (NMDA) receptors *(44–46)*. The above pathologic effects of homocysteine might be ameliorated by the administration of betaine (as a substitute for methyl donation by 5-methyl tetrahydrofolate in the conversion of homocysteine to methionine; Fig 1) or dextromethorphan (a noncompetitive antagonist at the NMDA receptor) *(47)*.

Chronic neurotoxicity may result from prolonged or repetitive insults caused by the above mechanisms. Clinically, it is characterized by loss of cognitive abilities, poor coordination, seizures, and learning disabilities. The pathologic hallmark of this syndrome is leuckoencephalopathy. A decrease or withdrawal of further insults to the CNS does not reliably prevent further deterioration or restore lost function. The best offense is a good defense: prevention by selection of the most effective regimen and identification of patients who may be at increased risk. For example,about 12% of people have a polymorphism of the enzyme methylenetetrahydrofolate reductase. The resultant decrease in enzyme activity causes mild hyperhomocysteinemia. Affected individuals, at increased risk for the development of venous thromboses, might also have a predisposition to develop MTX-related neurotoxicity *(48)*.

7. FUTURE CONSIDERATIONS

There are still large gaps in our understanding of folate biochemistry and its perturbation by antifolate drugs. In addition to the questions raised in this review, other areas require attention. First, MTX may not be the best classical folate analog. For example, aminopterin is a better substrate than methotrexate for folylpolyglutamyl synthase *(49)*, which might explain the greater toxicity of aminopterin in early studies. Alternative schedules of aminopterin could harness its increased activity while limiting concomitant toxicities. Second, the clinically relevant mechanisms underlying resistance to MTX are not well understood. Decreased expression of the reduced folate carrier, increased expression of DHFR, decreased affinity of MTX for DHFR, decreased expression of FPGS, and increased expression of γ–glutamyl hydrolase do not completely explain resistance to MTX *(50)*. Conversely, the increased sensitivity of patients with Down syndrome to MTX also is not well understood *(51)*. Finally, newer antifolate drugs may have different mechanisms for cellular uptake and different target enzyme specificities *(49,50)*. These agents could be used to treat (or to prevent) MTX resistance. The answers to these and related questions require well-designed clinical studies that include parallel pharmacologic and biochemical end points.

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ANTILEUKEMIC DRUGS

III

B

ASPARAGINASE

27 Asparaginase

BARBARA L. ASSELIN AND JOANNE KURTZBERG

CONTENTS

1. INTRODUCTION

The enzyme L-asparaginase (L-Asp) has been an important chemotherapeutic agent for the treatment of acute lymphoblastic leukemia (ALL) and all other lymphoid malignancies for over 30 yr. It has demonstrated efficacy in remission induction as well as subsequent phases of therapy *(1–7)*. In addition, L-Asp may have some activity in non-Hodgkin's lymphomas and acute myeloid leukemias, although these indications have not been well investigated *(8–11)*. L-Asp is unique in terms of its nature as an enzyme, potential for selective starvation of certain types of malignant tissues, mechanisms of drug resistance, and the lack of overlapping toxicities with other antineoplastic drugs. Unlike many chemotherapeutic agents, L-Asp does not enter cells, is not myelosuppressive, and usually does not cause mucositis, making it an ideal agent in combination chemotherapy regimens. Although L-Asp is recognized as a vital antileukemic drug, there are many unanswered questions regarding optimal dosing regimens, use of alternative forms, application of clinical pharmacologic studies, and understanding of treatment failures. With advances in biopharmacology, clinicians and researchers alike are taking a new look at therapy with L-Asp by examining pharmacologic end points and cellular effects of the enzyme on both normal and malignant tissues. Knowledge of the pharmacologic and cellular factors affecting L-Asp treatment is important for all those who treat patients with acute leukemia or related lymphoid malignancies.

In 1953, Kidd *(12)* reported his observation that normal guinea pig serum possessed antitumor activity against various transplanted lymphomas in rats and mice. Eight years later, Broome *(13)* demonstrated that the enzyme, L-Asp, was responsible for this antitumor effect. Mashburn and Wriston *(14)* successfully purified L-Asp from *Escherichia coli* and showed that this enzyme exhibited the same antitumor activity found with guinea pig serum, thus providing a readily available source of enzyme for preclinical and clinical studies. L-Asp has been isolated and characterized from various organisms, including many bacteria, as well as plants and the plasma of certain vertebrates *(15–19)*, but resources have never been directed toward development of a clinical grade drug for clinical studies of their activity or toxicity profiles. In the United States, three preparations of L-Asp are currently available for clinical use: (1) *E. coli* (Elspar); (2) the enzyme derived from *Erwinia chrysanthemi* (Erwinase); and (3) pegaspargase (PEG; Oncaspar), a modified form of the *E. coli* enzyme. The clinical and biochemical features of L-Asp have been summarized in several comprehensive reviews by recognized investigators in the field *(20–24)*. This chapter focuses on the salient details that define the state of the art, current controversies and questions, and goals for optimizing the uses of L-Asp. Specific questions to be discussed include

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Table 1
Biochemical Properties of Therapeutic Asparaginases

	E. coli		
	Native	*PEG*	Erwinia
Activity[a] (IU/mg protein)	280–400	280–400	650–700
K_m (μ*M*) L-asparagine	12	12	15
K_m (μ*M*) L-glutamine	3000	3000	1400
Ratio maximal activity L-Gln/L-Asp	0.03	0.03	0.10
Molecular weight (Daltons)	141,000	170,000	138,000

Abbreviations: L-Gln, L-glutaminase; L-Asp, L-asparaginase.
[a]One international unit hydrolyzes 1 μmol of asparagine per min.
Modified from ref. *24* with permission

(1) Which form of L-Asp is the best? (2) What is the optimal dose and schedule? (3) What drug interactions are important? and (4) Is laboratory-based monitoring important?

2. MECHANISM OF ACTION

L-Asp is an example of biologic therapy designed to exploit the identified nutritional differences between the host's somatic and malignant cells. As an enzyme, L-Asp hydrolyzes the amino acid asparagine, which is nonessential for normal human cells but essential for human lymphoblasts, into aspartic acid and ammonia, thus depleting the circulating pool of serum asparagine *(15,17,25,26)*. Leukemic lymphoblasts and certain other tumor cells that lack or have very low levels of L-Asparagine synthetase do not synthesize L-Asp *de novo (27–30)*. These cells normally rely on serum asparagine as their source for protein synthesis and survival. As a chemotherapy drug, L-Asp has a unique mechanism of action selectively starving the leukemic cells that are unable to synthesize adequate amounts of asparagine *de novo*. Cytotoxicity results from inhibition of protein synthesis and a secondary delayed inhibition of nucleic acid synthesis, blocking progression of the cell cycle in the G1 phase and preventing cell proliferation *(30–33)*. These effects ultimately result in cell death by induction of apoptosis *(31–33)*. L-Asp does not penetrate the central nervous system (CNS); however, asparagine from cerebrospinal spinal fluid (CSF) is depleted similarly to that in serum *(17,34–37)*. It diffuses into the intravascular space as a result of the concentration gradient from CSF to plasma and is immediately hydrolyzed. Thus, L-Asp administered systemically is also useful for CNS prophylaxis.

The contribution of glutaminase activity to the therapeutic effects of L-Asp is not known. It has been observed that the more glutaminase activity possessed by the L-Asp preparation, the broader the spectrum of antitumor activity observed *(22)*. As shown in Table 1, the available therapeutic asparaginases have different biochemical properties including different levels of glutaminase activity. The only enzyme properties that correlate with antitumor activity are K_m and half-life *(14,15,18)*. Thus far, the increased antitumor efficacy associated with glutaminase activity has not been observed in the clinical situation. It also has been postulated that glutaminase activity contributes to clinical toxicity associated with asparaginase therapy, particularly neurotoxicity and hepatotoxicity, as a result of glutamine depletion and glutamic acid accumulation.

3. MECHANISM OF RESISTANCE

Resistance to L-Asp is thought to result from several mechanisms. At the cellular level, it arises from tumor cell induction of asparagine synthetase synthesis. L-Asp-resistant cell lines have been found either to contain increased amounts of asparagine synthetase or to induce its synthesis in the presence of asparagine-depleted medium *(27,38)*. Similarly, in the only study examining the role of asparagine synthetase in the intrinsic resistance of human leukemia, leukemic cells from 18 patients who had never been treated with L-Asp had low levels of asparagine synthetase *(29)*. Of the nine patients treated with L-Asp and restudied, those with leukemia resistant to L-Asp responded with a sevenfold increase in leukemic cell asparagine synthetase activity, whereas those with a sensitive form of the disease did not. Although this intriguing study was performed on patients with various types of leukemia, the data suggest that L-Asp resistance is caused by induction of asparagine synthetase and/or selection of cells with high asparagine synthetase activity.

Host resistance is postulated to occur when the production of antibodies against L-Asp results in neutralization of enzyme activity or acceleration of enzyme clearance from the circulation *(17,26,39–49)*. Thus, clinical hypersensitivity or the phenomenon of silent hypersensitivity (e.g., antibody-mediated neutralization of L-Asp activity or clearance of L-Asp without clinical symptoms of an allergic reaction) may be an important previously unrecognized mechanism of drug resistance among patients receiving L-Asp therapy. The frequency of this "silent" inactivation or immune clearance of L-Asp as a mechanism of resistance remains to be explored *(43–49)*, but preliminary data suggest that this phenomena is present in >50% of patients with relapsed ALL and prior exposure to L-Asp in frontline therapy *(47–49)*. Development of resistance in tumor cells and/or the host by these or other mechanisms continues to limit the effectiveness of L-Asp in the clinical setting.

4. DOSE-LIMITING TOXICITIES AND THEIR MANAGEMENT

L-Asp has a distinct toxicity profile characterized primarily by immune-mediated hypersensitivity reactions and adverse events related to the inhibition of protein synthesis *(29,39,50–52)*. The most common dose-limiting factor in the use of L-Asp has been the development of hypersensitivity *(25)*, discussed below. In addition, normal tissues with high rates of protein synthesis (e.g., the liver, pancreas, and coagulation system) are most susceptible to non-hypersensitivity-mediated L-Asp toxicity. Since L-Asp is not toxic to the gastrointestinal or oral mucosa or bone marrow and to date has not been associated with late adverse effects, it can easily be added to combination chemotherapy regimens.

Most patients experience some evidence of chemical hepatotoxicity, typically manifest by decreases in serum albumin, fibrinogen, antithrombin III (AT III) or serum cofactors, serum lipoprotein levels, and (less frequently) an increase in serum liver transaminase levels and bilirubin. Hyperammonemia is an

almost universal laboratory finding that has no apparent clinical significance since it is only rarely associated with hepatoencephalopathy *(39,44,51)*. Hepatic function usually returns to normal when the drug is cleared or discontinued. Thus, it has been postulated that serum albumin, fibrinogen, or ammonia levels might serve as useful surrogate markers of L-Asp activity. Unfortunately, preliminary studies have not shown adequate correlations among direct measures of enzyme activity, asparagine depletion, and serum proteins to make this useful for clinical practice. Clinical hepatotoxicity is rarely dose-limiting, even when asparaginase is used in combination with other potentially hepatotoxic therapies such as methotrexate, mercaptopurine, anthracyclines, or vincristine. Initial concern regarding excessive hepatotoxicity associated with the PEG form of asparaginase has not been justified. Occasionally, hypoalbuminemia is severe, causing anasarca, and should be treated with albumin infusion. This does not require discontinuation of L-Asp therapy.

Disorders of the coagulation system evidenced by imbalances in the production of clotting factors (hypofibrinogenemia, decreased AT III, deficiencies of protein C or S, decreased von Willebrand factor, and others) are common side effects of L-Asp therapy *(53–59)*. Although L-Asp causes a decrease in most plasma coagulation proteins, AT III is affected to a greater extent than other hemostatic proteins *(55,57,59)*. Acquired AT III deficiency, well known as a complication associated with L-Asp therapy, predisposes to a hypercoaguable state. Although AT III deficiency is thought to play a primary role in the development of thromboembolic complications of L-Asp, studies suggest that other factors probably play a role *(56,58,60)*. Despite the high frequency of chemical abnormalities, bleeding episodes or thrombotic events are infrequently reported (1–3% in most series) and rarely require discontinuation of therapy *(61–63)*. If hemorrhage or thrombosis occurs, therapy with fresh frozen plasma (FFP) to replace deficient procoagulant proteins has been the standard of care in an effort to prevent recurrence during continuation of asparaginase therapy. However, in a study of eight children, the infusion of 20 mL/kg FFP was shown to have no clinically important or statistically significant effect on the levels of coagulation proteins *(57)*.

In the event of a CNS thrombosis, an attempt should be made to identify specific deficiencies of the natural anticoagulant proteins so that replacement therapy can be as specific as possible. When a thrombotic event is associated with AT III deficiency, treatment with the specific AT III concentrate, now available in the United States, should be considered *(64,65)*. Most often, treatment includes discontinuation of L-Asp and supportive measures with infusion of FFP to replace deficient hemostatic proteins, although the benefits of FFP have not been demonstrated. It is possible to restart L-Asp after allowing several weeks for neurologic recovery without risk of recurrence *(61–63)*. In making decisions regarding whether L-Asp should be continued after a CNS thrombosis, one must consider the importance of L-Asp to treatment of the patient's underlying malignancy against the small risk of recurrent stroke. Within the Dana-Farber Leukemia Consortium, patients have continued intensive weekly L-Asp therapy following a stroke without recurrence. Recently, an increase in thrombotic events, primarily catheter-related, has been observed and reflects, at least in part, the increasing use of implanted central venous access devices. Therapy for such thromboses should include standard anticoagulation with heparin, low molecular weight heparin, or coumadin until after both L-Asp therapy is complete and the catheter is removed. Temporary discontinuance of L-Asp can be helpful for 3–4 wk while anticoagulation therapy is being initiated and evidence of clot stabilization or regression can be documented.

Another important consideration is the documentation of underlying hereditary or congenital conditions that result in a hypercoaguable state such as protein C or S deficiency or a factor V Leiden mutation state prior to initiation of L-Asp therapy. These children have an even higher risk of thrombosis and therefore should be very closely monitored. At this time there are no data to support prophylactic anticoagulation when L-Asp is to be administered.

L-Asp can adversely affect both the endocrine (insulin-secreting) and exocrine (digestive enzyme-secreting) cells of the pancreas. Some patients develop signs and symptoms of diabetes due to decreased synthesis of insulin. Hyperglycemia may be more severe when L-Asp is administered in combination with prednisone, but the risk can be reduced if the L-Asp course is administered after the course of prednisone is completed *(2,50)*. Up to 15% of L-Asp-treated patients experience acute pancreatitis manifested by anorexia, nausea and vomiting, and abdominal pain *(2,6,50,66,67)*. Approximately 2–8% of children experience life-threatening clinical pancreatitis, which prohibits further exposure to the drug. An equally small percentage of patients develop transient hyperamylasemia with mild abdominal discomfort that spontaneously resolves over a few days and therefore is neither a dose-limiting complication nor a contraindication to further asparaginase therapy.

Since it was first introduced into clinical trials, L-Asp has been reported to cause abnormalities in lipid metabolism, most notably hypertriglyceridemia *(50,51,68,69)*. The mechanism for this phenomenon is related to increased endogenous synthesis of very low density lipoproteins (VLDL) and decreased lipoprotein lipase activity *(69)*. In a recent study examining L-Asp-associated lipid abnormalities, 67% of patients had fasting triglyceride levels >200 mg/dL during L-Asp therapy, and 19% had levels >1000 mg/dL *(69)*. None of the seven patients with a triglyceride level >1000 developed pancreatitis. In contrast, of the patients without triglyceride elevation, 9% developed pancreatitis. Although their findings suggest that modification of L-Asp therapy is not indicated for hypertriglyceridemia, the authors offer two important caveats. First, in the setting of hypertriglyceridemia >2000 mg/dL, the risk of pancreatitis may be enhanced owing to severe chylomicronemia. Second, in chylomicron-induced pancreatitis, serum amylase levels may be normal and abdominal pain may be the only marker of pancreatitis, making close monitoring of such patients imperative.

Nonspecific gastrointestinal toxicity (nausea, vomiting, and anorexia) is common in older children, teenagers, and adults treated with intensified asparaginase therapy. Supportive care

with nutritional supplementation and, in extreme cases, parenteral nutrition can be used to complete the full course of L-Asp therapy. Neurotoxicity (depression, lethargy, fatigue, somnolence, confusion, irritability, agitation, and dizziness) occurs in up to 25% of adult patients treated with L-Asp *(70)* but is less common in children. A relationship between high blood ammonia levels and either liver toxicity or cerebral dysfunction (e.g., encephalopathy) has not been established.

Extensive use of L-Asp in children does not appear to be associated with the development of late adverse effects. Patients receiving intensive asparaginase therapy as part of etoposide-containing regimens may be at increased risk of developing second leukemias induced by topoisomerase-targeted drugs *(71,72)*.

4.1. Hypersensitivity

Hypersensitivity, a result of immunologic sensitization to the foreign protein, is the most common dose-limiting toxicity seen in clinical practice with any of the three available L-Asp preparations. Clinical hypersensitivity reactions are reported in up to two-thirds of patients receiving intensive schedules of the native forms of the enzyme *(3,6,29,39,50,51,71)*. Since the observation by Nesbit et al. *(73)* of fewer allergic reactions when L-Asp is administered intramuscularly (compared with intravenously), this route of administration has become widely accepted. Notably, fewer allergic reactions are observed when L-Asp is used in combination chemotherapy rather than as a single agent. This effect is related to the immunosuppression that results from multiagent chemotherapy, blocking the immune response to L-Asp. The most significant risk factor for the development of hypersensitivity is repeated courses of treatment, particularly when there are breaks of more than a month between courses, such as during induction and delayed intensification *(6,52,71,74)*.

The most common clinical manifestation of hypersensitivity is urticaria; however, the spectrum of allergic reactions ranges from localized erythema at the injection site to systemic anaphylaxis. Grading of clinical hypersensitivity reactions is not necessarily consistent among treating physicians. The new National Cancer Institute Common Toxicity Criteria, Version 2.0, has the advantage of specifically differentiating between urticaria and bronchospasm as signs of allergy as well as using the duration of symptoms and need for treatment as a gauge of severity. Unfortunately, this system does not incorporate a localized reaction at the injection site as a sign of allergy. This is important since patients on combination chemotherapy may not exhibit classic symptoms of widespread urticaria and rarely have overt bronchospasm. In addition, this definition may not identify patients with pharmacologic evidence of allergy, since antibody production and altered pharmacokinetics may occur with minimal or no clinical symptomatology *(44)*. This definition does not identify patients with silent hypersensitivity.

Patients with hypersensitivity usually have IgG or IgE antibodies in their serum *(23,39,41,75)*, but more than half of patients develop anti-asparaginase antibodies without clinical evidence of hypersensitivity reactions, a phenomenon termed *silent hypersensitivity*. These antibodies may diminish L-Asp efficacy by neutralizing L-Asp activity and/or increasing the rate of clearance *(43–45)*. Therefore, even though L-Asp treatment might be safely continued, the antileukemic effect would probably be diminished. These findings suggest that the clinical practice of premedication with antihistamine prior to an L-Asp dose in order to prevent overt symptoms of allergy is not appropriate since the risk/benefit ratio is high. These patients are being exposed to the risk of adverse effects potentially without benefit of the desired biologic effect. This is one of the strongest arguments supporting the need for lab monitoring of pharmacologic end points during L-Asp therapy.

Investigators of the Dana-Farber Consortium and the Pediatric Oncology Group (POG) hypothesize that if a large enough group of children were followed longitudinally, a cohort of these patients would demonstrate decreased enzyme activity with repeated doses related to the appearance of anti-asparaginase antibodies without clinical signs of allergy. It is not possible from available data to estimate the incidence of such alterations in pharmacokinetics among patients on front-line studies. A phase II study, POG 8866, demonstrated that increased antibodies were associated with rapid clearance of enzyme activity *(46)*.

Antibody tests have limited value for predicting which patient will have an allergic reaction. Because of the frequency of severe allergic reactions early in the development of L-Asp, clinical investigators used skin tests in an attempt to predict reactions. These efforts were not successful because of their poor sensitivity and specificity *(51,76)*. Allergic reactions were observed in patients with negative skin tests, and positive skin tests were not always followed by clinically apparent allergy. Attempts at desensitization by administration of a series of small doses subcutaneously have been equally ineffective and generally do not alter antibody production *(76)*.

5. WHAT IS THE BEST L-ASP PREPARATION?

Historically, *E. coli* L-Asp is the most widely used and extensively studied of the L-Asp forms. Very early in the development of this drug, it became apparent that hypersensitivity was a common dose-limiting toxicity. Therefore, alternative forms of L-Asp that do not share antigenic crossreactivity and might prove less toxic were investigated. Today, L-Asp for clinical use is available in three preparations. In the United States, the *E. coli* product, commercially marketed by Merck as Elspar, is licensed for use in the therapy of patients with newly diagnosed or relapsed disease. *Erwinia*, the native preparation derived from *Erwinia chrysanthemi*, remains investigational and is only available to patients with allergy to the *E. coli* product (Ogden Bioservices, Speywood Pharmaceuticals). The *Erwinia* product is commercially available in Canada and Europe as Erwinase (marketed by Porton), and investigators in these countries have much more experience and data on the use of this preparation. A third preparation, PEG-L-asparaginase (nonproprietary name, pegaspargase) is a chemically modified form of the enzyme in which native *E. coli* L-Asp has been covalently bound to monomethoxypolyethylene glycol (PEG). This modification reduces the immunogenicity of this foreign protein and increases the serum half-life without interfering with enzymatic activity. Pegaspargase (available commercially

as Oncaspar) is approved by the Food and Drug Administration for use in combination chemotherapy for the treatment of patients with ALL who are hypersensitive to native forms of the drug. Properties of the three preparations are summarized in Tables 1 and 2.

Different L-Asp preparations are not readily interchangeable. Discussion of the best asparaginase to use in combination chemotherapy regimens must consider primarily three factors: (1) antitumor efficacy; (2) toxicity; and (3) pharmacology. The potential advantages and disadvantages of each preparation are listed in Table 3 with respect to these three characteristics.

All three of the currently available asparaginase preparations have demonstrated efficacy in the treatment of ALL *(1–7,16,46–49,77–82)*. For two decades the Dana-Farber Leukemia Consortium has conducted intensive, randomized studies of patients with newly diagnosed precursor-B-ALL to determine the relative efficacy and toxicity of different L-Asp preparations. Key studies from the Dana-Farber and other cooperative groups that are discussed below are outlined in Table 4.

As part of a clinical study, Protocol 87-01, conducted between 1987–1991, Asselin et al. *(82)* prospectively evaluated the in vitro and in vivo efficacy of these three widely used preparations. Children newly diagnosed with ALL were randomized to receive a single dose of *E. coli* asparaginase, *Erwinia*, or PEG on day 0 of a 5-d investigational window. All three types of L-Asp produced equivalent leukemic cell kill in both the in vitro and in vivo assays *(82)*. The equivalence of *E. coli* asparaginase and PEG was confirmed in a subsequent study, Protocol 91-01. As part of Protocol 91-01 (1991–1995), patients were randomized to receive either *E. coli* (25,000 IU/m^2/wk × 30 wk) or PEG (2500 IU/m^2 every other week for 30 wk). There was no difference in event-free survival based on L-Asp preparation (84% *E. coli* vs 78% PEG, $p = 0.29$) *(67)*. Of particular interest was the observation by these investigators that the 5-yr event-free survival of patients receiving <25 wk of asparaginase therapy was significantly worse than for patients receiving at least 26 wk of L-Asp (73% vs. 90%, $p < 0.01$) *(67)*. L-Asp intolerance was not associated with the initial type of preparation (PEG or *E. coli*). These data suggest that the dose intensity and duration of L-Asp therapy may be more important than the type of L-Asp in terms of disease control and long-term outcomes.

In Protocol 95-01 (1996–2000), patients were randomized to receive either *E. coli* or *Erwinia*, both dosed at 25,000 IU/m^2 weekly for 20 wk. Comparison of *E. coli*- and *Erwinia*-treated groups demonstrated more relapses in the patients randomized to *Erwinia* (p= 0.005), leading to the conclusion that *Erwinia* may be less efficacious than *E. coli* asparaginase when dosed once a week *(66)*. This finding stands in contrast to the results of a nonrandomized comparison of *E. coli* and *Erwinia* by Eden et al. *(85)* and a randomized study reported by Otten et al. *(86)*. They observed no difference in outcome in terms of disease-free survival with either product. A possible explanation for the lack of difference relates to the dosing schedules since both studies used short courses, 3 wk, of L-Asp during induction in combination with greater than five other agents. In this setting, L-Asp may have very little impact. The dosing interval, every 2–3 d, also serves to overcome the less favorable pharmacology of the *Erwinia* preparation. As discussed below, the pharmacology of L-Asp and dosing can have a significant impact on antileukemic efficacy. The decreased efficacy reported for the *Erwinia* product may be predicted by pharmacology and may be overcome by adequate dosing.

Table 2
Pharmacologic Characterization of Different L-Asp Preparations

	Half-life (mean d ± SD)	*Asparagine depletion (d)*
Naive patients[a]		
E. coli	1.28 ± 0.35	14–23
Erwinia	0.65 ± 0.13[b]	7–15
PEG	5.73 ± 3.24[c]	26–34
	Dose (IU/m^2)	*Half-life (mean d ± SD)*
Patients with prior hypersensitivity to *E. coli*[a]		
E. coli (n = 5 patients)	25,000	Undetectable
PEG (n = 5 patients)	2500	1.82 ± 0.3[d]
	Duration of PEG enzyme activity[e] *(mean d ± SD)*	
	Low antibody	*High antibody*
Patients previously treated with *E. coli* and/or *Erwinia*		
Hypersensitive	13.3 ± 0.6	4.0 ± 1.4
Nonhypersensitive	12.2 ± 1.4	6.0 ± 0.0
L-Asp naive	13.8 ± 1.5	Not applicable

[a]Adapted from ref. *44* with permission.
[b]Significantly shorter than *E. coli* ($p < 0.001$).
[c]Significantly longer than *E. coli* ($p < 0.001$).
[d]Significantly shorter than value for patients without history of hypersensitivity ($p < 0.01$)
[e]PEG dosing schedule for all patients was 2500 IU/m^2 every 14 d. Data from refs. *46–49*.

Toxicities occur with similar frequencies with all three available L-Asp preparations, with the exception of decreased allergic reactions observed with *Erwinia* and PEG *(66,67)*, as shown in Table 5. Overall, L-Asp therapy was well tolerated. Hypersensitivity was the most frequent adverse event in each group, and there were no significant differences in the incidence of pancreatitis, hepatitis, or coagulopathy, including stroke, among the three treatment groups. In these studies, *Erwinia* and PEG appeared to be less toxic than *E. coli* asparaginase, primarily due to a reduction in mild allergic events *(66,67)*.

Most importantly, studies have shown that following allergic reactions to the *E. coli* preparation, either *Erwinia (77,78, 87)* or PEG *(46,80,81)* can be safely substituted. Not surprisingly, patients with a history of allergy to one form of the enzyme are more likely to suffer an allergic reaction with a substituted enzyme as well. Interestingly, older patients (9–18 yr old) are more likely to experience L-Asp-related toxicity (except hypersensitivity), in comparison with patients younger than 9 yr of age *(67)*.

The most significant difference between the three products relates to pharmacologic properties, specifically the kinetics

Table 3
Relative Advantages and Disadvantages Associated with Use of the Three L-Asparaginase Preparations

	Advantages	*Disadvantages*
E. coli	Most extensively studied Least expensive per dose	Not all products have same activity (Elspar vs Medac vs Crasnitin)
Erwinia	Fewer side effects than *E. coli* or PEG Can be used if allergy to *E. coli* or PEG occurs	Short $T_{1/2}$→frequent dosing required, frequent clinic visits Probably less effective Not licensed in U.S.
PEG	Fewer dose-limiting allergic reactions since less immunogenic Can be used if allergy to *E. coli* or *Erwinia* occurs Long $T_{1/2}$→less frequent dosing, fewer clinic visits Possibly better efficacy Overall less costly when substituted for *E. coli* by pharmacoeconomic analysis	Expensive, cost 20× more than *E. Coli* and 2× more than *Erwinia* Large volume of injections (sometime 2 per dose) since only available at present concentration Longer half-life can increase intensity of toxic or hypersensitivity reactions

Table 4
Selected Studies in Development of the Three L-Asparaginase Preparations in Patients with ALL

Study (ref.)	*Design*	*L-Asp dose*	*Key findings*
DFCI Consortium			
77-01 *(5)*	Randomization to L-Asp vs no L-Asp during intensification	*E. coli* 25,000 IU/m^2/wk`im (total, 20 doses)	Improved EFS with L-Asp treatment, 63% vs 44% no L-Asp ($p = 0.05$)
87-01 *(44,82)*	Investigational window randomization to 3 L-Asp preparations (single dose)	*E. coli* 25,000 IU/m^2, *Erwinia* 25,000 IU/m^2, PEG 2500 IU/m^2—all im on d 0	Equivalent in vitro and in vivo leukemic cell kill for all three preparations Comparative pharmacology studies (*see*Table 2) Prognostic significance (in vitro response related to EFS)
91-01 *(67)*	Randomization to *E. coli* vs PEG during intensification	*E. coli* 25,000 IU/m^2/wk, PEG 2500 IU/m^2/14 d, im (total, 30 wk)	Equivalent EFS rates Equivalent toxicity except decrease in mild allergy with PEG (*see* Table 5) Prognostic significance (L-Asp intolerance→decreased EFS)
95-01 *(66)*	Randomization to *E. coli* vs *Erwinia* during intensification	*E. coli* 25,000 IU/m2/wk, *Erwinia* 25,000 IU/m^2/wk im (total, 20 doses)	Increased relapse rate, decreased EFS with *Erwinia* Less toxicity, less allergy with *Erwinia* (*see* Table 5)
2000-01 (Sallan, S. E., unpublished data)	Randomization to standard vs. individualized dose during intensification	*E. coli* 25,000 IU/m^2/wk im *E. coli* 12,500 IU/m^2/wk im with dose adjustments based on L-Asp activity (total, 30 doses)	Open to accrual
Pediatric Oncology Group			
8866 *(46)*	Randomization to *E. coli* vs PEG induction for relapsed patients	*E. coli* 6000 IU/m^2/im 3×/wk for 9 doses, PEG 2500 IU/m^2/im every other wk (× 2 doses)	PEG safe in patients with prior allergy to *E. coli* Silent hypersensitivity 63% at study entry High antibody titer associated with poor outcome
9310 *(81)*	Randomized PEG doses induction for patients with 1st marrow relapse	PEG 2500 IU/m^2 im/wk vs every other wk	CR rate 97% in weekly PEG group High L-Asp levels associated with a better CR rate High antibody titer associated with lower L-Asp level and lower response rate
8704 *(71)*	Randomization to *E. coli* vs no L-Asp during intensification for T-All	*E. coli* 25,000 IU/m^2/wk im (total, 20 doses)	Improved DFS with *E. coli* High rate of second leukemias 12% in *E. coli* group
Childrens Cancer Group			
1962 *(83)*	Randomization to *E. coli* vs PEG during intensification	*E. coli* 6000 IU/m^2 im 3×/wk, times 9 doses, PEG 2500 IU/m^2 im, single dose	Faster rate of remission in PEG-treated group Serum L-Asp levels >0.03 IU/mL important Persistence of L-Asp levels >0.03 IU/mL more frequent with PEG
1941 *(84)*	PEG pharmacology study, early marrow relapse	PEG 2500 IU/m^2 im on d 2 and 16 during induction	Asparagine depletion (< 3μ*M*) significantly correlated with response rate Serum L-Asp level and antibody titer did not predict asparagine depletion or induction response

Abbreviations: EFS, event-free survival; CR, complete remission; DFS, disease-free survival; DFCI, Dana-Farber Cancer Institute.

Table 5
L-Asparaginase-Related Toxicities According to Preparation Administered[a]

	E. coli		Erwinia	*PEG*
Toxicity	*A* *(n = 108)*	*B* *(n = 144)*	*B* *(n = 136)*	*A* *(n = 120)*
Patients with ≥ 1 toxic event (%)	36	24	9	24
Allergy (%)	21	16	6	13
Mild (no. patients)	20			9
Severe (no. patients)	1			4
Pancreatitis (%)	7	6	2	8
Mild (no. patients)	1			3
Severe (no. patients)	6			5
CNS thrombosis/bleeding (%)	1	5	2	2
Other thrombosis (%)	1		Not studied	3
Other toxicity[b]	8	2	1	4
Dose-limiting (%)	12	9	5	10
Death (no. of patients)	0	0	0	1 (pancreatitis)

[a]*A*: *E. coli* vs PEG, patients with ≥ 1 toxic event, $p = 0.06$; mild allergy, $p = 0.02$; overall allergy, $p = 0.12$, no other significant differences. Data from ref. *67*.
B: *E. coli* vs *Erwinia*, patients with ≥ 1 toxic event, $p < 0.001$; allergy, $p = 0.01$; no other significant differences. Data from ref. *66*.
[b]Headache, hyperglycemia, hypoproteinemia, hyperlipidemia, nausea/vomiting, hypertransaminemia.

of enzyme activity and duration of asparagine depletion *(44)*, as shown in Table 2. Pharmacokinetic investigations within the Dana-Farber Leukemia Consortium (Protocol 87-01) revealed that the apparent $T_{1/2}$ of enzyme activity was dependent on the enzyme preparation used. As observed by previous investigators, the $T_{1/2}$ of *Erwinia* is significantly shorter than that for the standard *E. coli* preparation ($p < 0.001$) *(44,75,88)*. The serum half-lives of patients treated with PEG was almost 5 times greater than the half-life of the native *E. coli* enzyme ($p < 0.0001$) *(23,44,79)*. Of note, L-Asp activity was measurable (>0.03 IU/mL) in the sera of patients receiving PEG for the entire 26-d observation period but had disappeared by d 8 and 13 for the *Erwinia* and *E. coli* enzymes, respectively *(44)*. As predicted by differences in half-life, the pharmacologic effect of L-Asp therapy, measured as the duration of asparagine depletion, was also significantly different for each preparation ($p < 0.01$) *(89)*. Similar findings were also reported by a group of M.D. Anderson investigators as part of a phase I study in adults *(23,42)*.

Based on pharmacologic parameters, PEG would appear to be the best L-Asp preparation. Because it is given less often, fewer injections and fewer clinic visits are required. In addition, a pharmacoeconomic assessment of PEG vs *E. coli* Asp by Peters et al. *(90)* suggests that PEG is less expensive than the *E. coli* product when substituted into standard ALL regimens. The increased cost of PEG is balanced overall by the decreased frequency of doses and decreased charges for clinic visits. *E. coli* is equivalent to PEG except for its pharmacologic profile, which requires more frequent dosing, making it less convenient and less ideal than PEG. One word of caution relates to the clinical toxicity occurring after PEG administration. Since the half-life of this preparation is longer than that of unmodified L-Asp forms, the duration of clinical hypersensitivity reactions, hypoproteinemia, coagulopathy, and pancreatitis may be more severe and longer in duration.

Erwinia L-Asp, based on currently available data, has the least favorable efficacy and pharmacologic profile, making it the third choice in frontline therapy. Adjustments in dosing, such as an increase in dose with a decreased dosing interval, may result in an improved pharmacologic effect and thus better antileukemic effects, but these predictions have not been tested.

Even though PEG appears to be the first choice, among available L-Asp forms, there is a significant disadvantage to incorporating it routinely into frontline therapies, that is, once a patient develops allergy to PEG they cannot receive *E. coli*, so that *Erwinia* becomes the only asparaginase available to them. Given the observation that asparaginase intolerance may be associated with a poor outcome, this caveat raises concern that a subset of patients will be unable to complete a full course of asparaginase therapy. By using *E. coli* in first-line treatments, one could safely and feasibly switch to PEG should hypersensitivity reactions develop and then subsequently to *Erwinia* if a second hypersensitivity reaction is encountered.

6. WHAT IS THE BEST DOSE AND SCHEDULE?

Despite almost 30 yr of clinical experience with L-Asp, the optimal dosage and schedule of asparaginase remains undefined. Unmodified *E. coli* and *Erwinia* enzymes have been used at a variety of doses and on multiple dosing schedules, including daily, every other day, and weekly. Advances in methods to study serum and cellular pharmacology stimulated a new look at L-Asp therapy over the past decade.

Using remission rates and incidence of toxicity, especially allergy, as the criteria for the best dose, multiple investigators *(1–6,8–11,26,52)* demonstrated that (1) L-Asp used in combination with vincristine and prednisone yielded response rates

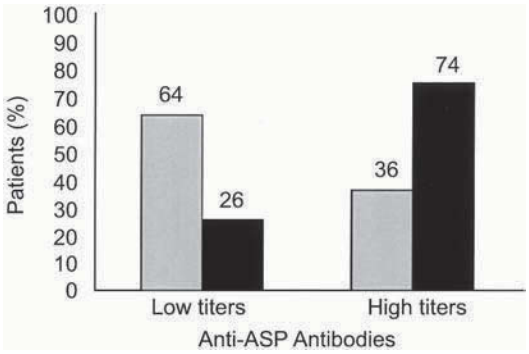

Fig. 1. Response rates as a function of anti-asparaginase (ASP) antibody titers. The percentages of patients with no response are indicated by black bars and those with a complete or partial remission by gray bars. (Modified from refs. *46* and *93* with permission.)

of 50–70% at all doses in patients with multiply relapsed ALL; (2) response rates were related to the number of previous asparaginase exposures rather than asparaginase dose; and (3) the incidence of hypersensitivity was less with lower doses, daily schedules, and in combination with other chemotherapeutic agents. Ertel et al. *(4)* were the only group that observed a dose-response relationship. Using asparaginase 3 times a week by im injection at doses of 300, 3000, 6000, and 12,000 IU/m,2 they obtained complete remission rates of 9.5, 35, 53, and 62.5%, respectively. Intermittent dosing three times a week became the accepted schedule when unmodified asparaginase was used in combination with vincristine and prednisone, since response rates were similar, toxicity was equivalent, and the schedule permitted less frequent clinic visits. The efficacy of this schedule in patients with newly diagnosed ALL was confirmed by Ortega et al. *(2)*, who administered L-Asp at 6000 IU/m^2 im 3 times per week for 9 doses, in combination with vincristine and prednisone, to 815 evaluable patients. The complete remission rate was 93%, which compared favorably with historical controls receiving vincristine and prednisone only, with only six episodes of hypersensitivity reported.

In a landmark study reported by Nesbit et al. *(73)*, patients were randomized to receive L-Asp alone at 12,000 IU/m^2 on Mondays and Wednesdays and 18,000 IU/m^2 on Fridays by either im or iv injection. They found that the im route was as effective *(25)* (response rate, 38% vs 41%) but less toxic than (hypersensitivity reactions, 0% vs 23%) the iv route. As a result, im injection is the preferred route in pediatric oncology centers.

The importance of L-Asp during intensification therapy was demonstrated by the Dana-Farber Leukemia Consortium in a randomized trial conducted between 1977 and 1979 *(5)*. The patients treated with L-Asp at 25,000 IU/m^2 weekly × 20 doses, in addition to the backbone intensification therapy, had significantly improved event-free survival (63% vs 44%, $p = 0.05$) compared with the group treated without L-Asp. In 1987, the POG conducted a randomized trial to evaluate the efficacy of a similar high-dose L-Asp intensification in patients with newly diagnosed T-cell ALL or advanced lymphoblastic lymphoma. This trial confirmed the improved disease-free survival for patients treated with asparaginase compared with those treated without the enzyme *(71)*.

Currently, the most commonly used dosing schedules are either 6000 IU/m^2 3 times per week or 10,000 IU/m^2 2 times per week for 2 to 3 wk as part of induction therapy as well as delayed intensification regimens [POG, Children's Cancer Group (CCG), and Berlin–Frankfurt–Münster Group (BFM)]. Since 1981, all patients treated according to Dana-Farber Consortium protocols have received a single dose of asparaginase during induction followed by 20–30 weekly injections of im asparaginase during intensification. These doses have been shown to be effective and tolerable when *E. coli* asparaginase is being used *(6,67,91,92)*. *Erwinia* is traditionally administered according to the same dose and schedule as *E. coli*, although there are no data to support this practice. Most notable is that none of these regimens are based on pharmacologic measures, and similar dose finding studies have not been performed for *Erwinia* L-Asp. Multiple investigators have observed significant differences in pharmacologic profiles of alternate preparations, as shown in Table 2, suggesting that the clinical practice of substitution of different asparaginase forms according to the same dosage regimen is not pharmacologically sound or effective.

PEG is most often given at a lower dose than either *E. coli* or *Erwinia*, on an every-other-week schedule based on the results of the following studies *(23,42,46,79,80)*. In a phase I dose-escalation study of PEG in adults (doses of 500–8000 IU/m^2 given iv every 2 wk), there was no relationship between the dose and efficacy or toxicity; however, the maximum tolerated dose was not reached *(23,42)*. During a phase II trial in which PEG was dosed at 2000 IU/m^2 im every 2 wk, the response rate was 30% on d 14 when PEG was used as a single agent and 61% on d 35 following multiagent therapy, including PEG *(80)*. The results of this study showed that PEG was effective during reinduction therapy when administered every 2 wk compared with three times per week for native preparations.

A randomized trial comparing the safety, efficacy, and feasibility of PEG vs native *E. coli* asparaginase as part of a standard induction regimen in children with ALL in second relapse was conducted by the POG study 8866 from 1988 to 1992 *(46)*. All children enrolled on the study had been exposed to native L-Asp. Of the 74 evaluable patients, 35 without prior hypersensitivity to asparaginase were randomized to treatment with either PEG (2500 IU/m^2 im every 2 wk) or native *E. coli* asparaginase (10,000 IU/m^2 im three times per week) in combination with a standard 28-d induction regimen of weekly vincristine and daily prednisone. Thirty-nine patients with a history of hypersensitivity were not eligible for randomization and were directly assigned to treatment with PEG-asparaginase in combination with vincristine and prednisone. The overall complete response rate was 40%, with no significant differences among the three treatment groups. In this study, however, patients with accelerated clearance of PEG (antibody-mediated) were less likely to respond to therapy (Fig. 1). No unexpected serious adverse reactions were seen in the patients treated with PEG-asparaginase, and, in general, nonallergic asparaginase-related toxicities were similar among all three treatment groups.

The lack of any new or unusual toxicity in the POG 8866 study led to the evaluation of the biweekly dosing schedule of PEG-asparaginase in patients newly diagnosed with ALL in frontline pilot studies conducted by the POG, the Dana-Farber Leukemia Consortium, and the CCG in the early 1990s. Investigators in the POG observed increased toxicity specifically related to asparagine depletion, including pancreatitis and hypoproteinemic syndromes with extreme weight loss, in the group of asparaginase-naive patients treated on the biweekly dosing schedule of PEG-asparaginase. Particularly affected was a subset of patients with high-risk disease on prolonged PEG-asparaginase therapy in combination with intermediate-dose iv methotrexate, iv 6-mercaptopurine, and iv higher dose cytarabine (J. Kurtzberg, unpublished data). Despite the increased nonallergic toxicity, fewer hypersensitivity reactions were observed compared with historical controls who had received native asparaginase preparations as frontline therapy.

The L-Asp preparation-dependent pharmacokinetics observed in these studies provide important information for establishing an appropriate dosing schedule for asparaginase. The longer $T_{1/2}$ following administration of PEG-asparaginase affirms the less frequent dosing schedule for this preparation; however, the shortened $T_{1/2}$ of the *Erwinia* preparation compared with the *E. coli* preparation suggests that the accepted dosing schedule for *Erwinia* asparaginase, which is identical to that for *E. coli* asparaginase, might not be optimal. More recent studies of PEG-asparaginase pharmacology suggest that the every-other-week schedule may not be ideal when PEG is being used in patients previously treated with the *E. coli* drug because of the potential for shortened half-life, probably due to silent hypersensitivity. In POG study 9310, the safety, efficacy, and pharmacology of L-Asp were compared in children with relapsed ALL randomized to PEG asparaginase (2500 IU/m^2) administered weekly vs every other week in combination with a standard induction regimen (prednisone, vincristine, doxorubicin, and L-Asp with triple intrathecal therapy) *(81)*. Of the 128 evaluable patients, the overall CR rate was 97% in patients who received PEG-asparaginase weekly and 82% in patients who received this preparation once every 2 wk (p= 0.02). As demonstrated in Fig. 2, response significantly correlated with mean plasma asparaginase levels (p= 0.01). The results of this study suggest that PEG administered weekly may be superior to the enzyme administered once every other week in relapsed patients and that plasma asparaginase levels correlate with response rate.

It has been shown previously that the in vivo clearance rate of asparaginase and its affinity for asparagine (K_m) are two important properties that predict the efficacy of the specific enzyme as an antitumor agent *(13,14)*. Given what is known about the available enzyme forms and our abilities to quantify enzyme activity and pharmacologic affect, future clinical studies should allow definition of a therapeutic dose or threshold. In order to maximize the therapeutic index of L-Asp, the optimal dose and schedule of treatment should be determined on the basis of pharmacologic testing rather than clinical criteria alone.

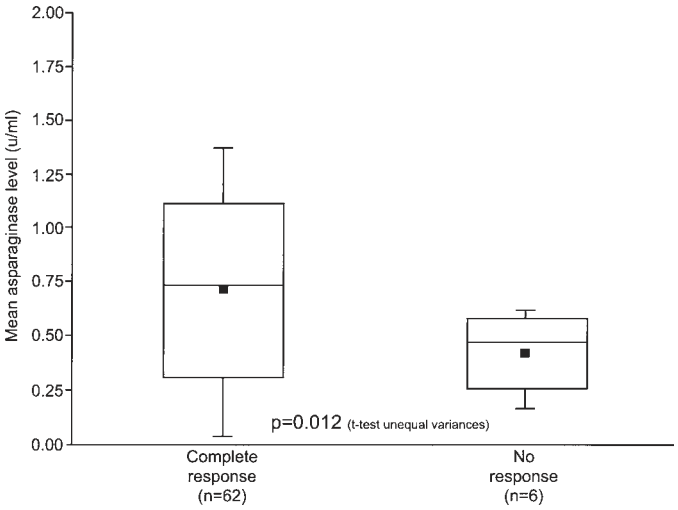

Fig. 2. Box-and-wisker plots of the distribution of mean asparaginase levels for patients with a complete response (M1 marrow) vs no response (M2 or M3 marrow) at the end of induction. The mean value of each group is represented by a closed square; the median value is indicated by the horizontal line within each box; and the top and bottom lines of the box delineate the 95% confidence interval. (From ref. *81* with permission.)

7. IMPORTANT DRUG INTERACTIONS

L-Asp has a unique toxicity profile that does not overlap with traditional antileukemic drugs, does not include myelosuppressive or intestinal effects, and is thus a favorable agent for use in combination chemotherapy. When used in combination with vincristine and prednisone, L-Asp is both effective and tolerable. An additional benefit of combining L-Asp with other immunosuppressive chemotherapeutic agents is the decreased frequency of hypersensitivity reactions, presumably through suppression of the patient's immune response to the foreign protein.

The combination of methotrexate and L-Asp is an example of two effective drugs that interact in a novel way to produce a synergistic effect on tumor cells with antagonistic inter-actions on normal cells. The schedule-dependent cytotoxic synergism between methotrexate and L-Asp was first demonstrated in vitro in murine and human leukemia cells by Capizzi et al. *(94–96)*. The optimal interval between L-Asp and subsequent methotrexate to maximize the synergistic effects was determined to be 10 d *(96)*, a time when lymphoblasts are recovering from the protein synthesis inhibition and the cell cycle blockade at G1 caused by L-Asp. As these recovering cells move in synchrony to the S-phase and initiate DNA synthesis they are particularly susceptible to the S-phase-specific DNA synthesis, inhibition of methotrexate. Interestingly, L-Asp was observed to antagonize the effects of methotrexate in L5178Y cells in vitro when given immediately before the antifolate, but aborts its actions when given in the 24 h after methotrexate *(95,96)*. In 1985, Jolivet et al. *(97)* reported that L-Asp decreases the metabolism of methotrexate to polyglumate forms, resulting in lower levels of intracellular drug. They suggested that this was the likely mechanism of the observed protective effect of L-Asp given after methotrexate. As a result, multiple investigators have used

the combination of methotrexate and L-Asp very successfully in clinical regimens, in which L-Asp acts as a "rescue" agent and permits the administration of otherwise toxic doses of methotrexate *(96,98–100)*.

The combination of cytarabine (ara-C) and L-Asp has been shown to be more effective than either drug alone in the treatment of murine and human leukemias *(1,101–105)*. Studies conducted in vitro and in vivo using the murine leukemia L5178Y cell line demonstrated a significant schedule-dependent synergy between high dose ara-C and L-Asp *(105)*. These experiments illustrated that the optimal sequence of drug treatment is ara-C followed by L-Asp and that the time interval between drugs was also important. The maximal synergistic benefit of this combination is achieved when the asparaginase is administered prior to cell recovery from ara-C-induced cytotoxicity, that is, within several hours of the ara-C dose. Schwartz et al. *(105)* propose several mechanisms for this synergy, such as L-Asp inhibition of the reinitiation of DNA synthesis, increased retention time of activated ara-C metabolites (ara-CTP), and reduction of deoxycitidine triphosphate (dCTP) levels, all of which may play a role. Subsequent clinical trials have demonstrated this same schedule-dependent enhancement of the therapeutic effects of L-Asp by ara-C, without a concomitant increase in toxicity to normal organs in patients with refractory acute leukemias *(106,107)*. This combination has been particularly effective for patients with refractory acute myeloid leukemia.

8. LABORATORY MONITORING

Studies have indicated that conventional chemotherapy sometimes fails because patients receive inadequate doses of drugs, not because their leukemia is drug-resistant. In a randomized trial of individualized methotrexate dosing, Evans et al. *(108)* showed that increasing the methotrexate dose in patients with rapid clearance of the drug significantly improved outcome without increasing toxicity in treatment of children with ALL. Similarly, in studies of L-Asp, multiple investigators have reported variable clearance, variable host tolerance, and the development of host resistance, all of which limit the drug's effectiveness. The previously cited studies in relapsed patients demonstrate a clear correlation between clearance and clinical response. Thus, the rationale for L-Asp laboratory monitoring and eventually individualized dosing regimes is based on the following observations: (1) the effect of pharmacology on outcome, particularly altered pharmacokinetics or pharmacodynamics (Figs. 1 and 2) *(46,80,81,83)*; (2) the high degree of interindividual variation in pharmacologic measures *(75,88,109)*; (3) the impact of dose intensity and L-Asp intolerance on outcome *(67,81)*; and (4) decreased toxicity when dose is appropriately lowered *(88,109,110)*. Pharmacologic studies of L-Asp have focused primarily on three laboratory end points: enzyme activity, depletion of L-Asp, and the development of anti-L-Asp antibodies.

As previously described, multiple investigators have confirmed that the pharmacologic characteristics of L-Asp, in terms of the clearance of enzyme activity and ability to deplete serum asparagine, depend on the nature of the enzyme used. The pharmacokinetics and pharmacodynamics of L-Asp are significantly altered in most but not all patients who develop anti-L-Asp antibodies, regardless of their clinical status, a phenomenon termed silent hypersensitivity. Such data suggest that silent hypersensitivity results in suboptimal asparagine depletion and diminished efficacy for a subset of patients. The POG 8866 and POG 9310 studies are unique because they are the only ones that examined the correlation between altered pharmacokinetics and outcome *(46,81)*. Although response rates in POG 8866 were not significantly different among the three treatment groups (overall response, 55–61%), a subset of patients with high IgG antibody titers cleared L-Asp more rapidly and had a response rate of 26%. Those with low titers cleared enzyme activity more slowly and had a significantly higher response rate, 64% (Fig. 1; $p = 0.02$). The absence of prior clinical hypersensitivity did not necessarily correlate with low antibody titers. Sixty-three percent of patients without a history of clinical hypersensitivity to L-Asp had high titers at study entry. All patients who cleared L-Asp rapidly developed high titers of antibody after their first PEG dose. Similarly, in POG 9310, there was a highly significant difference in complete remission rates between weekly (69/71, 97%) and biweekly (60/73, 82%) PEG dosing ($p = 0.003$). Hypersensitivity was rare (6/144, 4%). Lower L-Asp levels were associated with high antibody titers. The complete remission rate was significantly associated with higher levels of L-Asp ($p = 0.012$). These results illustrate (Fig. 2) that the presence of antibody and/or low serum L-Asp levels predicts a lower response rate. Therefore, development of an antibody response to L-Asp, which is not necessarily predictable from the patient's past clinical history, is associated with decreased efficacy.

Colleagues from the CCG have provided preliminary data on first-line induction therapy with a single dose of PEG (2500 IU/m^2) vs *E. coli* (6000 IU/m^2) given three times per wk for nine doses. They concluded that a dose of PEG produced a faster rate of remission ($p = 0.05$) and more persistence of L-Asp activity than did multiple doses of the native enzyme *(83)*. It appeared to be important to achieve trough enzyme levels of > 0.03 IU/mL. Relatively low levels of L-Asp could be related to either an inadequate dosing schedule or a short serum half-life, with or without antibody production. These data confirm the findings of Dana-Farber and POG investigators that L-Asp pharmacokinetics and pharmacodynamics are directly related to the drug's antileukemic efficacy. By contrast, CCG study 1941 of patients with early marrow relapse found that L-Asp enzymatic activity did not predict asparagine depletion, and neither serum enzymatic activity nor antibody titer predicted induction response. However, asparagine depletion was significantly correlated ($p = 0.03$) with response rate in this population of previously exposed patients. It appeared to be important to achieve a trough serum asparagine level of <3 μM *(84)*. Thus, the ideal strategy to optimize dosing would be to measure at least periodically one of the three pharmacologic end points mentioned above.

As suggested by all the studies described earlier, there is a significant degree of interpatient variability in pharmacologic measures following treatment with any of the L-Asp preparations, especially in patients with a history of prior exposure. High interindividual variability was most clearly indicated in a

study of pharmacokinetic dose adjustment by Vierra-Pinheiro et al. *(109)*, who report a wide range of mean L-Asp trough levels in 21 children on study, with a coefficient of variation as high as 77%. During intensification therapy, Asselin et al. (unpublished data) have also observed a significant variation in trough levels (range, 0.05–0.4 IU/mL). This patient-to-patient variability has been observed with all the preparations and with a variety of doses. The significance of intrapatient variability is less well defined because sample sizes in such studies have been so small. Boos et al. *(75)* have reported that three of five children treated with *Erwinia* had inadequate asparagine depletion after repeated exposures *(75)* Asselin et al. (unpublished data) studied nine children throughout a 20-wk course of *E. coli* and found no change in enzymatic activity. Therefore, the only means to ensure adequate dosing of individual patients receiving asparaginase is to perform intermittent monitoring.

Dose intensity has been recognized as an important feature of successful cancer treatments *(7)*. Most recently, Dana-Farber investigators observed that duration of L-Asp therapy had a significant impact on disease-free survival in Protocol 91-01 *(67)*. Patients receiving more than 25 of the 30 planned weekly doses fared significantly better than patients receiving less than 25 doses (disease-free survival, 90% vs 73%; $p < 0.01$). In this study, L-Asp intolerance was a significant negative prognostic factor. It has also been observed that some toxicities of L-Asp, particularly hypofibrinoginemia, thrombotic events, hyperglycemia, and hypoalbuminemia, can be ameliorated by lowering the prescribed dose, thus permitting the continuation of L-Asp therapy *(110)*. Unfortunately, pancreatitis, the most frequent dose-limiting toxicity after hypersensitivity, is unlikely to be a dose-related phenomenon. Careful laboratory monitoring to ensure that a pharmacologically effective dose is maintained is advised.

Although many investigators would agree that laboratory monitoring of L-Asp pharmacology would be useful, most could not agree on which of the three laboratory parameters would be best. Enzyme activity can be determined on unprocessed serum by several routine laboratory methods and therefore is the simplest, least costly and most practical of the measurements to perform. Asparagine measurement requires rapid deproteinization of the serum or some other method to inactivate L-Asp activity ex vivo and provide access to high-performance liquid chromatography. These requirements make evaluation of asparagine depletion unsuitable for widespread application. Antibody titers usually done by enzyme-linked immunosorbent assay are less convenient and the least sensitive to changes in the pharmacologic effect of L-Asp. Thus far, measurement of L-Asp activity is the most reproducible and fastest to perform, and early studies have found that it correlates with asparagine depletion and possibly clinical outcome.

Periodic measurement of enzyme activity, asparagines, and/or antibody levels in patients treated according to a variety of L-Asp schedules will allow further characterization of intra- and interindividual variability, the relationship between dose and effect, and silent hypersensitivity. Another hurdle is to determine what specific levels are clinically significant and therefore equivalent to "adequate doses." Future studies must test whether targeting of pharmacologic end points will positively influence disease outcome. Another important goal of monitoring studies would be to improve L-Asp tolerability by individualizing dosing strategies. Thus, a laboratory monitoring program using pharmacologic end points to define the best dosing strategies is vital to optimizing L-Asp treatment regimens.

Despite numerous clinical trials of L-Asp, two unanswered questions are essential to defining the optimal use of this enzyme: (1) What is the critical level of asparagine depletion necessary to achieve the antileukemic effect of L-Asp? (2) What is the critical level of L-Asp activity necessary to maintain adequate asparagine depletion? Treatment with L-Asp aims at depleting serum asparagine in order to exhaust the substrate supply that is essential for leukemic cell viability. Critical to this aim is the determination of the critical level of asparagine depletion or duration of depletion that is necessary for antileukemic efficacy. In other words, how much depletion is sufficient to starve the leukemic cells? The answer to this question is not known, although several hypotheses based on biochemical estimates and some in vitro data have been proposed. Asparagine-requiring cells can be maintained in cell culture with an asparagine concentration of 10 μM (1×10^{-5} *M*) *(39,111)*. Normal serum asparagine levels are 40 μ*M*, more than sufficient to supply the amino acid for cell needs. Since the K_m of the *E. coli* and *Erwinia* enzymes for asparagine is 10 μ*M* (1×10^{-5} *M*) *(16,112)*, considerable excess L-Asp is required in serum to degrade asparagine to sufficiently low concentrations to halt leukemia cell growth. Holcenberg et al. *(113)* predicts that serum enzyme activity levels > 0.03 IU/mL are needed to maintain asparagine depletion *(113)*. A study of PEG pharmacodynamics in rhesus monkeys by Berg et al. *(35)* demonstrated that serum L-Asp activity > 0.1 IU/mL was necessary to maintain asparagine depletion, whereas Ricciardi et al. *(34)* postulated that a serum activity level of 0.1 IU/mL would reliably deplete asparagine in both serum and CSF.

9. FUTURE STUDIES

Evidence has accumulated in recent years of host factors that affect antileukemic drug efficacy or ameliorate drug toxicity *(108)*. These data support the treatment strategy of individualized dosing as a means of optimizing antileukemic therapy. L-Asp is an ideal drug for such an approach since three preparations are available for clinical use, assay methods for measurement of several pharmacologic end points have been established, antibody-mediated hypersensitivity potentially prevents drug efficacy in some patients, and some toxicities appear to be dose-related. Whether individualized or pharmacologically based dosing regimens will translate into improved therapeutic outcomes remains to be tested.

Recently, the finding of the *TEL/AML1* gene rearrangement (which results from a translocation involving chromosomes 12 and 21) as the single most frequent chromosomal alteration in childhood ALL has attracted considerable attention. Pieters et al. *(114)* have observed an increased in vitro sensitivity of leukemic cells to the antileukemic effect of L-Asp with the *TEL/AML* abnormality. The mechanism of this sensitivity has not been elucidated. Their data suggest the intriguing notion

that this population of patients, identifiable by the presence of *TEL/AML* in their leukemic cells, will particularly benefit from optimization of L-Asp therapy. Such hypotheses will need to be tested in the clinic in the future.

To overcome the limitations associated with L-Asp therapy (including allergy, rapid clearance, induction of cellular resistance, and dose-limiting toxicities), several approaches have been tried. In addition to the successful covalent linkage of PEG, investigators have tried a variety of enzyme manipulations ranging from isolation of asparaginase enzymes with more *(115,116)* or less *(117,118)* glutaminase activity; polymerization with albumin *(119)*; gel entrapment of the enzyme *(120)*; encapsulation into red blood cells *(121)* or liposomes *(122)*. Enzymes with both glutaminase and asparaginase activities in preclinical testing have shown a broader spectrum of antitumor activity, but their development in the clinical arena has been limited by increased toxic side effects *(113,116)*. It is postulated that an asparaginase enzyme with no glutaminase activity would be less toxic, but these enzymes have not yet been tested in clinical trials. Kravtzoff et al. *(121)* have shown that the use of red blood cells as carriers of L-Asp greatly improved the half-life and pharmacodynamic parameters of the drug in comparison with the free enzyme. Internalization of L-Asp into liposomes was shown to prolong circulation time and also enhance antitumor activity in animals *(122)*. These formulations of L-Asp therapy are candidates for future development.

In summary, 30 years of experience has demonstrated that L-Asp is a vital component in the treatment armamentarium of lymphoproliferative disorders, especially ALL. The beauty of this enzyme as a drug relates to the potential ability to document a positive pharmacologic effect in individual patients. Dose adjustment based on individual pharmacologic profiles is the promise of the future.

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28 Asparaginase Treatment

CARMELO RIZZARI

CONTENTS

1. INTRODUCTION

Almost 50 yr ago Kidd *(1)* discovered that the progression of murine lymphomas was halted by the injection of guinea pig serum. Ten years later, Broome *(2)* found that the L-asparaginase (L-Asp) enzyme was responsible for this antitumoral activity, and since then, it has been classified as an anticancer drug. Over the years following its discovery, L-Asp was shown to be capable of inducing complete remissions of short duration in acute lymphoblastic leukemia (ALL) *(3,4)*, even as a single agent. L-Asp has thus been used as a key element of multiagent chemotherapy schedules for the treatment of ALL and non-Hodgkin's lymphoma *(5–14)*. Although this enzyme has been widely investigated in clinical trials and in bio-chemical and clinical pharmacology studies *(15,18)*, many challenging issues regarding its use remain controversial. Basic science and clinical investigators are still seeking to optimize L-Asp treatment and to devise better means of monitoring the drug's biologic effects, to overcome the onset of resistance, and to prevent side effects. This chapter summarizes the most relevant pharmacologic, clinical, and toxicity data on L-Asp, mainly obtained from European investigators of diverse L-Asp products.

Since commercially available L-Asp products are prepared by a number of different companies and each displays distinct pharmacokinetic and pharmacodynamic characteristics, this chapter indicates the products by their commercial names (whenever this information is available). Table 1 provides information on the L-Asp products that have been or are being used in clinical practice.

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2. HOW DOES L-ASP WORK?

The main biologic effect of L-Asp is a rapid and prolonged fall of asparagine plasma levels, achieved by the hydrolysis of this amino acid into aspartic acid and ammonia. Interestingly, even in the presence of high quantities of these two hydrolysis compounds, no negative feedback on L-Asp enzymatic activity is induced *(19–21)*. Asparagine is an essential amino acid for leukemic blasts, which (unlike normal cells) have inefficient asparagine synthetase activity and are thus unable to synthesize adequate amounts of asparagine for their survival. During L-Asp treatment, external sources (i.e., plasma levels) of asparagine are removed, resulting in progressive and substantial impairment of protein and DNA/RNA synthesis, which ultimately induces leukemic cell death *(22,23)*. This mechanism of action provides the basis for a highly selective activity against lymphoid blast cells, whereas the drug's limited immune- and myelosuppressive activity allows it to be used in the context of intensive and myelosuppressive chemotherapy schedules. In addition to this cytotoxic effect, other mechanisms such as apoptosis may be involved in clinical efficacy *(24,25)*. Relevant mechanisms of resistance to L-Asp treatment may be (1) the induction of asparagine synthetase activity in lymphoid blasts, resulting in increased production of intracellular asparagines; or (2) the development of neutralizing antibodies, with

Table 1
L-asparaginase Products: Current Commercial Competition[a]

Medicinal product (bacterial source)	*Pharmaceutical company*
L-asparaginase medac®, Pasoral®, Kidrolase®, Leunase® (*E. coli*)	Medac, Kyowa Hakko, Roger Bellon/Aventis, Christiaens, Nycomed
Elspar® (*E. coli*)	Merck
Oncaspar® (*E. coli*-pegylated)	Medac, Rhône-Poulenc Rorer
Erwinase® (*Erwinia chrysanthemi*)	Edzon, Ipsen-Speywood

[a] The production of the *E. coli* preparation of Crasnitin was discontinued by Bayer in the early 1990s.

increased clearance of the drug, reduced L-Asp activity levels, and an inadequate degree and duration of asparagine depletion.

Glutamine (Gln) is considered an L-Asp second substrate. This amino acid is transformed in glutamic acid and ammonia. Although the deaminating activity of L-Asp on Gln is not as high as that on asparagine, Gln depletion is considered an additional mechanism for some toxic effects related to the use of L-Asp. In fact, since a steep fall in asparagine levels is obtained just a few minutes after L-Asp administration, elevated amounts of Gln present in plasma become available and are hydrolyzed by the enzyme. Elevated concentrations of aspartic and glutamic acid (degradation products together with ammonia of asparagine and Gln deamination) are found in the plasma of subjects undergoing L-Asp treatment and are believed to be involved in the toxic side effects arising from use of L-Asp *(26,27)*.

3. WHICH L-ASP PRODUCT IS THE BEST?

Since the recognition of its antitumor activity, L-Asp has been derived from many bacterial sources. The two most used forms of native L-Asp in clinical practice derive from two different bacterial species (i.e., *Escherichia coli* and *Erwinia chrysanthemi*). These two products have different amino acid sequences, pharmacokinetic characteristics and antigenic properties *(28–30)*. For example, with intramuscular administration at a dosage of 25,000 IU/m^2, Erwinase was reported to have disappeared from plasma much faster than the *E. coli* product Elspar, with mean half-lives of 0.65 ± 0.13 and 1.24 ± 0.17 d, respectively *(31)*. Nonetheless, Erwinase has generally been used at the same dosage and schedule as *E. coli* L-Asp products, particularly as second-line treatment in patients showing clinically relevant allergic reactions to the *E. coli* enzyme. In fact, these two native products are considered to be equally immunogenic but to have only limited cross-immunogenicity *(32)*, which makes it easy to shift from one product to another when a hypersensitivity reaction occurs *(33–35)*. Erwinase has often been used as the first-choice drug in frontline ALL trials, with reports of a lower incidence of side effects and apparently superimposable clinical efficacy (historical comparisons), compared with the *E. coli* product Crasnitin *(36–39)*. In the late 1980s, a new *E. coli* L-Asp product, derived from a different strain, was introduced under one of the following trademarks: Asparaginase medac, Kidrolase, or Leunase (Table 1); it replaced Crasnitin, which was no longer commercially available in the early 1990s. Dosages and schedules were not modified, since it was assumed that these preparations had superimposable biologic and clinical effects compared with Crasnitin. Later on, several countries established comparative drug monitoring programs, with the main objective of understanding better the pharmacokinetic/pharmacodynamic profiles and/or patterns of toxicity and/or efficacy of the different L-Asp preparations available on the market.

In a pharmacologic study investigating childhood ALL patients treated with Erwinase, within the framework of the Italian Association for Pediatric Hematology and Oncology (AIEOP) ALL'91 study, asparagine plasma depletion was observed in the vast majority of cases exposed to conventional doses of Erwinase (10,000 IU/m^2 im q3d × 8) during the first exposure (i.e., induction phase), contrasted with only a minority of cases during the second (reinduction phase) or third (maintenance phase) exposure. This was true with either conventional or high (25,000 IU/m^2 im weekly × 20) doses of the same product, suggesting that the 7-d interval between one dose and the next was too long. Good correlation between plasma and cerebrospinal fluid (CSF) asparagine levels was also observed, although further evidence that high-dose Erwinase may be less effective when given for a second or third time was highlighted by the fact that some cases that were undepleted at d 7 did not even deplete 3 d after administration. An *ad hoc* experiment revealed enzyme inactivation in the plasma of those patients in whom asparagine depletion was not found *(40)*. These data concurred with other findings from patients treated according to the same protocol, showing that asparagine depletion in the CSF was obtained in 75% of cases, 3 d after the administration of conventional doses of Erwinase during induction (first exposure) and in only 25% of cases 5 d after administration of high-dose Erwinase during reinduction/maintenance phases (second exposure) *(41)*.

Mechanisms involved in this phenomenon, although not fully elucidated, may depend on drug pharmacokinetics (e.g., increased clearance of L-Asp after repeated treatments), increased tissue production of asparagine, and the presence of "silent" inactivating factors in the plasma (neutralizing antibodies), as observed in the course of treatment with the *E. coli* L-Asp products *(42)*. Other investigators have recently reported similar results on depletion of CSF asparagine in patients treated with Elspar in the context of a different chemotherapy schedule (frontline and relapse protocols) *(43)*. Although L-Asp does not cross the blood-brain barrier, a good correlation exists between plasma and CSF asparagine levels, making L-Asp a potentially effective drug for central nervous system (CNS)-directed treatment in ALL.

Boos et al. *(44)* compared pharmacokinetic data of patients undergoing L-Asp treatment during the induction or reinduction phase of the ALL-Berlin–Frankfurt–Münster (BFM)-

90 study. Treatment included Crasnitin or medac L-Asp during induction (10,000 IU/m^2 given iv at 3-d intervals × 8) and reinduction (four identical doses given every 3–4 d). In case of allergy, Erwinase was used for this second exposure. Surprisingly, during the induction phase, medac L-Asp proved to have very different pharmaceutical properties, displaying a longer half-life than that of Crasnitin and significantly higher L-Asp median trough plasma levels (475 IU/L vs 74 IU/L); however, asparagine depletion was satisfactory in both groups. During the reinduction phase, plasma L-Asp levels in the medac group were superimposable to those found during induction, with >90% of samples displaying complete asparagine depletion; for children treated with Crasnitin or Erwinase, inadequate L-Asp activity levels and asparagine depletion were found in a substantial proportion of patients *(44)*. Consequently, a dose adjustment study under pharmacokinetic control was carried out on patients undergoing the same BFM chemotherapy schedule, in an attempt to optimize the treatment schedule for patients requiring second exposure treatment with Erwinase, after a first exposure to medac L-Asp (induction). An increased dose of 20.000 IU/m^2 × 9 (given every other day on a Mon/Wed/Fri schedule during the reinduction phase) ensured adequate L-Asp levels and asparagine depletion for a 3-wk period, comparable to those observed with the no-longer-marketed Crasnitin given at the traditional schedule of 10,000 IU/m^2 q3/4d × 4. However, even at this increased dosage, high interindividual variability and the silent inactivation phenomenon were observed in some patients *(45)*.

More recently, patients enrolled in the European Organization for Research and Treatment of Cancer (EORTC) 58881 study (based on a BFM backbone), were randomized to receive either the *E. coli* product Kidrolase or Erwinase *(7)*, during induction, at the same dosage (10,000 IU/m^2 q3d × 8 doses). Patients treated in the Kidrolase arm showed an increased rate of coagulation disturbances but a highly significant difference in terms of disease-free survival in comparison with patients treated in the Erwinase arm. However, possible interactions between the first study mentioned above and the additional two distinct prospectively randomized studies conducted within the framework of that trial, have somehow complicated the interpretation of the afore-mentioned results.

An excess of enzymatic activity due to the use of intensive L-Asp schedules or, alternatively, of more potent products, may also lead to increased toxicity. Patients undergoing medac L-Asp treatment at a dosage of 10,000 IU/m^2 during induction and reinduction of the BFM-ALL-90 study experienced more frequent disruption of coagulation parameters than that observed when Crasnitin was used in the same phases *(46,47)*. In line with this experience, the use of Leunase, whose properties are identical to those of medac L-Asp, given intramuscularly every other day for nine doses at a dosage of 10,000 IU/m^2 in the context of a different chemotherapy induction schedule (with respect to the BFM backbone), was poorly tolerated and resulted in excessive toxicity *(48)*.

A dose reduction study during induction was started in 1994 in children included in the German ALL-BFM-95 study, with the main objective of maintaining adequate asparagine depletion by using the medac L-Asp preparation at a dosage lower than 10,000 IU/m^2. The targeted L-Asp activity level was 100 IU/L. Two steps of dose reduction (5000 and 2500 IU/m^2) were studied. Adequate asparagine depletion was observed in the vast majority of the samples analyzed in both reduction steps. Median L-Asp activity levels were 265 IU/L in the 5000 IU/m^2 group and 102 IU/m^2 in the 2500 IU/m^2 group. CSF asparagine levels were undetectable in all cases. However, high inter- and intraindividual variability was seen with the 2500 IU/m^2 dosage, and so medac L-Asp was accordingly used at 5000 IU/m^2 *(49)*, for the induction schedule of the subsequent ALL/non-Hodgkin's lymphoma (NHL)-BFM trials.

Another study to evaluate L-Asp activity (in plasma) and asparagine depletion (in plasma and CSF) was undertaken in children with ALL exposed for the first time to Erwinase or medac L-Asp (given im or iv at the conventional dosage of 10,000 IU/m^2, q3d × 8) during the induction phase of the BFM-based AIEOP ALL95 study. Plasma and CSF asparagine trough levels were undetectable (i.e., <0.2 μ*M*/L) in all cases, including those with L-Asp trough activity levels of <50 IU/L. However, L-Asp trough activity levels during the administration of medac L-Asp were significantly higher compared with those of Erwinase. Asparagine depletion was obtained in virtually all patients without relevant differences seen between the im or iv administration routes. As a result, the dosage of medac L-Asp during the induction phase was subsequently changed to 5000 IU/m^2, also in the AIEOP ALL 95 study, whereas the dosage of 10,000 IU/m^2 for patients treated with Erwinase remained unchanged. The evidence that asparagine levels may be undetectable even in patients with L-Asp trough activity levels of <50 IU/L has challenged the opinion that an activity level of at least 100 IU/L is required to obtain adequate asparagine depletion *(50)*.

L-Asp treatment optimization must take into account the specific pharmacokinetic characteristics of the preparation used and the possibility that clinically undetectable immunologic reactions that the host develops against this foreign protein can determine a loss of enzymatic activity and resistance to L-Asp treatment, even when the drug is used at high doses.

With regard to the native forms, a definite reduction of immunogenic properties, an increase of nonreactivity to antibodies, a significantly longer half-life (5.73 ± 3.24 d), and a reduction of the number of injections for the patient (i.e., also fewer hospital admissions) have been obtained by conjugating the native molecule to polyethylene glycol (PEG) *(31)*; because of these characteristics, the PEG product has mainly been used in patients hypersensitive to the native products *(51)*. Patients who have presented with a hypersensitive reaction to native forms of L-Asp demonstrate a decreased half-life for both native and PEG L-Asp preparations, but the half-life of PEG L-Asp lasts longer than that of native products *(31)*. These patients could benefit from an increased dosage and/or a more frequent dosing schedule.

More recently, PEG L-Asp has been also used in ALL front-line chemotherapy schedules for both adults and children *(52–54)*. A study on ALL children treated within ALL-BFM-95 was recently conducted to determine whether a single dose (1000 IU/m^2 iv) of Oncaspar (medac) administered during reinduction (second exposure) could reduce the 30% rate of

allergic reactions (with subsequent shift to Erwinase) expected to happen in that phase with the native medac L-Asp. Furthermore, the study sought to maintain the targeted L-Asp activity levels of 100 IU/L (and thus adequate serum asparagine depletion) for 2 wk and of ≥ 50 IU/L for 3 wk (thus comparable to levels obtained with four doses of native medac L-Asp given at 10,000 IU/m^2 or with nine doses of Erwinase given at 20,000 IU/m^2). No allergic reaction was observed among the 66 investigated nonhypersensitive children. Other toxicities were as expected. A targeted L-Asp activity level of 100 IU/L was obtained for 14 d in about 70% of patients. However, a rapid decline of L-Asp activity levels was observed at d 21 in the remaining 30% of patients. "Silent inactivation" may therefore occur after administration of the PEG L-Asp product, and L-Asp activity monitoring aimed at early identification of subjects with inadequate activity levels (i.e., presenting silent inactivation) seems necessary even for the PEG L-Asp product when used as second exposure *(55)*.

Another pharmacologic study was recently carried out in the United Kingdom on a limited number of children with relapsed ALL (and heavily pretreated with Erwinase) to evaluate whether the use of Oncaspar (medac), given subcutaneously at a dose of 1000 IU/m^2, would allow adequate L-Asp activity levels (i.e., 100 IU/L) for 14 d. The toxicity pattern was as expected. Surprisingly, the desired L-Asp activity levels were achieved in all of these intensively pretreated patients for a median period of 19 d (range, 14–24) *(56)*. It is unclear whether this favorable pharmacologic profile would have been different if patients had been pretreated with an *E. coli* L-Asp product instead of Erwinase.

Recent data from the CCG randomized study 1962 of Oncaspar (Rhône-Poulenc Rorer; one dose given at 2500 IU/m^2 im) vs Elspar (three weekly doses at 6000 IU/m^2 im for 3 wk) in standard-risk ALL children shows that the toxicity profiles of these preparations are very similar and that Oncaspar was able to induce a more persistent L-Asp activity and a faster rate of complete hematologic remission *(53)*. Asselin et al. *(57)* found a correlation between the in vitro and in vivo antileukemic effect determined by the early application of three different L-Asp preparations (*E. coli*, *Erwinia*, and PEG L-Asp) and that the absence of response was of prognostic significance *(57)*. It is not yet clear, however, whether PEG L-Asp administration in newly diagnosed patients since their first exposure to the drug is able to decrease the allergic reactions rate and silent hypersensitivity during both the first or further exposures and, as a consequence, to increase L-Asp therapeutic efficacy.

Many different factors may thus make one L-Asp preparation "better" than another. If L-Asp preparations are not used according to their specific antigenic, pharmacokinetic, and pharmacodynamic properties, very different patterns of efficacy and toxicity may be observed. Other factors that may also greatly contribute to these effects are dosage, administration route, number of previous exposures, frequency of administration, interval between a previous treatment cycle, and other drugs administered concomitantly.

4. IS LABORATORY MONITORING NECESSARY?

L-Asp is an important drug in the armamentarium of anticancer treatment; however, its specific value as a single agent is difficult to assess in modern, multiagent chemotherapy schedules. L-Asp effects are closely related to the specific pharmacologic and pharmacodynamic characteristics of the preparations available for clinical use; monitoring specific pharmacologic end points is considered a useful way of trying to maximize therapeutic efficacy and minimize side effects. L-Asp activity levels and asparagine depletion are the main biologic correlates of the L-Asp therapeutic effect; a reliable assessment of serum L-Asp activity levels and plasma/CSF asparagine levels should therefore be considered of primary importance for L-Asp efficiency monitoring. Furthermore, since the production of neutralizing antibodies following repeated exposures to the drug may significantly alter the intrinsic pharmacologic characteristics of the preparation used, immunologic assays could also be used to refine the dose schedule or switch to a different L-Asp product. Tests used to assess the aforementioned pharmacologic end points are only briefly outlined at this point in the chapter, whereas clinical implications related to laboratory monitoring are discussed in some detail.

Methods for measuring L-Asp activity levels are not simple and generally consist, after some specific enzymatic reactions, of the spectrophotometric quantification of absorbance variation occurring as a result of NADPH or NADH oxidation *(57)* or ammonia release *(43,57–59)*. Very recently, the fluorometric assessment of 7-amino-4-methylcoumarin release was proposed as a rapid and reliable assay for L-Asp enzymatic activity evaluation *(60)*. When serum and CSF were investigated in monkeys and humans undergoing L-Asp treatment, it was found that L-Asp levels of approx 100 IU/L were able to deplete asparagine completely in the CSF and in the serum *(61)*. These activity levels have mostly been considered the optimal target of some dose-monitoring studies *(45,49)*. However, results of another recent study, previously reported in this chapter, seem to indicate that adequate asparagine depletion may be obtained *(51)*, even in the presence of L-Asp activity levels of <50 IU/L. Exceedingly high L-Asp activity levels do not seem to be needed for adequate therapeutic efficacy achievement and may increase toxicity *(46–48)*.

Determination of asparagine levels (and of other amino acids) in serum is generally performed by using a reverse-phase-high-performance liquid chromatography technique following precolumn derivatization with o-phthaldialdehyde and fluorescence detection *(44,50,51)*. An important technical problem raised in the past has been that measurements of asparagine levels might have been overestimated owing to the residual activity of L-Asp present in blood drawn from patients *(59)*; significant amounts of asparagine could have been hydrolyzed by L-Asp still present in the sample during blood centrifugation and even after storage of the plasma at –80°C. The use of L-Asp inhibitors such as 5-diazo-4-oxo-norvaline was proposed to overcome this problem *(59)*. Unfortunately, the inhibitors used have proved to be highly unstable and to disturb the analytical procedure of determining asparagine levels. A procedure based on the rapid deproteinization of blood by adding sulphosalycilic acid has also been used *(45,58)*. However, in our experience these procedures are not needed to measure plasma asparagine levels, at least when L-Asp activity is measured too *(50)*. The optimal level of

asparagine depletion is still a matter of debate. In normal subjects, plasma asparagine levels range from 40 to 80 μ*M*. In the past, Boos et al. *(44)* have used different degrees of plasma asparagine depletion with the aim of evaluating this phenomenon more uniformly: complete depletion, nearly complete depletion, moderate reduction, slight reduction, and no reduction, yielding plasma asparagine levels of <0.1 to >40 μ*M (44)*. Because of the quantification limit of the assays currently adopted, an asparagine level of ≤0.2 μ*M* may be considered a reliable indicator of adequate depletion.

Anti-L-Asp antibody concentration is usually measured by an enzyme-linked immunosorbent assay procedure *(62)*, including an assay for the measurement of specific IgG concentrations directed against different commercial L-Asp preparations *(63)*. The usefulness of antibody measurement as an outcome predictor is controversial *(42,64,65)*. A significantly increased risk of developing hypersensitive reactions in children with high-titer anti-L-Asp antibodies was recently reported *(62)*. Since it seems that the use of L-Asp products with higher biologic activity is associated with better clinical efficacy *(7)*, a pharmacologic monitoring system aimed at maintaining therapeutic plasma L-Asp levels represents a reasonable approach to treatment optimization in clinical trials. This information, together with asparagine levels and anti-L-Asp antibody titer determinations, may therefore offer a wealth of information that could lead to a more rational schedule design and to maximal therapeutic efficacy.

5. WHAT IS THE BEST SCHEDULE AND ADMINISTRATION ROUTE?

5.1 Scheduling

L-Asp is a valuable component of combination chemotherapy for ALL and NHL. Since the beginning of its clinical use, L-Asp has been used in a variety of treatment schedules and with widely ranging doses and time intervals between single doses and treatment phases. On the basis of pharmacokinetic data, it is commonly believed that the longer enzymatic activity is maintained in the plasma, the better the depletion effect of asparagine levels in plasma and CSF obtained and the higher the blast cell killing expected *(22,61)*. In multiple-drug chemotherapy schedules for induction of hematologic remission, L-Asp dosing generally lasts for 3 wk. Even though "standard doses" (6–10,000 IU/m^2 at 2–3-d intervals) are generally given during induction, this chapter has already highlighted the fact that use of a specific product *(7)* or the adoption of different intervals may lead to very different toxicity and efficacy patterns *(46–48)*. Longer treatment periods, during both induction and intensification phases, have also been applied with favorable results *(5,6)*.

A recent hypothesis is that children with B-lineage ALL bearing the *TEL-AML1* rearrangement owing to the t(12;21) clonal translocation may be more effectively treated with regimens containing high-dose L-Asp (generally weekly administrations of 25,000 IU/m^2) *(66,67)*. In a study by the Pediatric Oncology Group (POG), however, a similar approach did not provide any benefits for B-precursor ALL patients *(10)*. In another POG study, three Elspar doses (10,000 IU/m^2) were given during induction to all patients, who, 3 mo after diagnosis (concurrently with the initiation of the continuation phase of therapy) were randomly assigned to receive or not receive the same product at high doses (25,000 IU/m^2/wk im × 20 wk). This schedule proved to be effective for a large cohort of children with T-ALL and advanced lymphoblastic lymphoma. In the experimental arm, however, an elevated number of failures was observed owing to secondary malignancies, possibly caused by an interaction between L-Asp and epipodophyllotoxins *(9)*.

The design of the AIEOP ALL 9102 randomized study for intermediate risk patients was based on the concept that protracted administration of high-dose L-Asp could be considered the ideal type of treatment intensification for a BFM-based schedule, given its proven efficacy, particularly as seen in the Dana-Farber Cancer Institute (DFCI) studies *(5,6,11)*, and its limited hematologic toxicity. However, in this AIEOP study, no significant difference in terms of disease-free survival was observed between patients randomized to receive or not receive a protracted high-dose Erwinase schedule (25,000 IU/m^2 im per wk × 20). The lack of efficacy of high-dose Erwinase in this study could be explained in different ways: the specific pharmacokinetic characteristics of the drug (the dose interval may have been too long for high-dose administration of the drug), the occurrence of inactivating factors of immunologic origin after repeated exposures, which may not have allowed sustained asparagine depletion in the plasma and CSF, and/or the already intensive treatment adopted in the study protocol (BFM-backbone) *(68)*. In the BFM-ALL-90 study (conducted in Germany during the same period as the AIEOP ALL 91), intermediate-risk patients were randomized to receive or not receive high-dose medac L-Asp (25000 IU/m^2 q2wk × 4) during the consolidation phase (i.e., after each high-dose methotrexate cycle infusion). Again, no difference was found in the two arms when disease-free survival rates were compared *(12)*. The adoption of intensive and/or protracted L-Asp administration schedules (particularly with higher doses or products with higher biologic activity) may thus improve results in some specific settings *(5,6,9,66)*, whereas in the context of other treatment strategies, theoretical advantages may be obscured by the intensity of other effective antileukemic agents *(10,12,68)* and/or by severe side effects *(46–48)*.

The design of an optimal L-Asp dosage and schedule should take into account different variables (e.g., preparation to be used, possible drug interactions, chemotherapy intensity for each treatment phase, and ALL subtypes to be treated). In particular, L-Asp should be administered with as few interruptions as possible either between single doses within the same treatment phase or between subsequent treatment courses. This could limit allergic reactions and minimize asparagine synthetase gene upregulation in leukemic blasts, thus further increasing the main cytotoxic mechanism of L-Asp. A routine change from one L-Asp preparation to another may reduce the incidence of allergic reactions *(55)* but, because of the possibility of silent inactivation onset, may also lead to an undetectably reduced efficacy of the drug *(55,65)*.

5.2. Route of Administration

Several investigations have been carried out to evaluate L-Asp efficacy and toxicity patterns according to the adminis-

tration route. Some studies have revealed the same efficacy but a higher incidence of severe anaphylactic reactions in patients treated by the iv route compared with the im route *(69–71)*. Comparative pharmacokinetic data regarding plasma trough activity levels after a first exposure to Erwinase or to the medac L-Asp given iv or im, show that these two administration routes provide equivalent pharmacologic effects in patients treated with the same product and suggest that the administration route should not affect clinical efficacy *(50)*. Nonetheless, a large proportion of patients undergoing a second or subsequent exposure to an L-Asp preparation, especially when given iv, will experience severe allergic reactions *(12,45,70)*. Erwinase has been reported to display a high specific activity that minimizes the amount of foreign protein given at each administration; this property could explain the lower incidence of immunologic reactions observed following Erwinase administration *(36–39)*. However, a significantly increased incidence of clinically relevant allergic reactions may be expected for the Erwinase product when the iv route is used, especially for the second exposure to the drug *(71)*. Furthermore, it is well known that the incidence of allergic reactions in patients treated with Erwinase due to a previous onset of allergy to an *E. coli* L-Asp product, is significantly higher than in patients exposed only to Erwinase and without reactions *(32)*.

An important issue to be taken into account regarding the "optimal" administration route is the patient's own perspective. The physical pain and the psychological distress caused by the repeated im injections normally planned in L-Asp administration schedules should not be underestimated. When different administration routes are considered equivalent in providing therapeutic and side effects, patients or their relatives should receive comprehensive information on the possibility of choosing the treatment modality.

6. WHAT DRUG INTERACTIONS SHOULD BE CONSIDERED?

L-Asp administration is generally used concomitantly with many other antineoplastic drugs that, *per se*, display a wide range of therapeutic and toxic effects. In particular, the use of steroids may enhance the risk of coagulopathy *(72)*. Drug interactions with antimetabolites are very peculiar. L-Asp administration before methotrexate (MTX) may not only lead to reduced formation of MTX polyglutamates but may also inhibit cell cycle progression toward the critical S-phase, when cells are highly sensitive to this drug *(73,74)*. However, cells refractory to MTX just after L-Asp administration may become more sensitive to MTX when it is administered 10 d after L-Asp. Conversely, administration of L-Asp immediately after MTX injection (i.e., 24 hr) permits an improved tolerance for cells undergoing this antimetabolite treatment. This concept has been translated into a chemotherapy schedule that, when administered in repeated cycles, has proved effective and only mildly toxic for childhood ALL patients *(75,76)*.

Clinically relevant interactions between L-Asp and epipodophyllotoxins have been reported. An unexpectedly high frequency of severe infections and mortality was observed in patients treated with Leunase (10,000 IU/m^2 three times/wk for 3 wk) in the framework of an induction treatment including vincristine, prednisolone, etoposide, and cytarabine. One possible explanation for this phenomenon was that the intensive use of Leunase could have decreased the systemic clearance of etoposide, thus indirectly enhancing myelosuppression and facilitating the occurrence of severe infectious complications *(48)*. Besides an increased systemic exposure to etoposide, prolonged exposure to L-Asp treatment may lead to impairment of the production of enzymes necessary for adequate DNA repair; these two biologic mechanisms could offer a possible explanation for the increased incidence of secondary leukemias observed in childhood ALL patients treated with chemotherapy regimens that include contemporary intensive use of these two drugs *(9,77)*.

7. WHAT ARE THE DOSE-LIMITING TOXICITIES?

7.1. Allergic Reactions and Silent Inactivation

Since L-Asp is a foreign protein, different types of allergic reactions represent highly relevant adverse effects. Immunological reactions against L-Asp may vary from enzyme inactivation without clinical reaction (silent inactivation) *(40,49,51)* to mild or severe local or generalized allergic reactions, or even anaphylactic shock. Deaths caused by severe allergic reactions are, however, very rare. The incidence of allergic reactions ranges from 5% to over 70%, depending on the duration of treatment, the number of prior exposures, the product used, the administration route, the concomitantly administered medications, and the interval between one exposure and the next. It has been hypothesized that contamination of the product by endotoxins partly explains this hypersensitivity pattern *(69)*. Allergic reactions limit L-Asp therapy, with switches from one product to another or, in some cases, drug discontinuation. When the development of antibodies occurs without a clinically relevant reaction, it is agreed that a silent inactivation is under way; in particular, this is observed in patients undergoing a second or further exposure to the drug and can involve any product given at any dosage and in the frame of any treatment schedule.

Cheung et al. *(42)* were able to demonstrate that a silent production of neutralizing antibodies occurred in some patients treated with an *E. coli* L-Asp product and that a correlation between the production of antibodies and prognosis could also be demonstrated.

Kurtzberg et al. *(65)* have reported that the half-lives of native *E. coli* L-Asp and PEG-L-Asp were much shorter in reacting patients (who also had high titers of anti-L-Asp antibodies), than in nonreacting patients. The appearance of high-titer anti-L-Asp antibodies was also associated with poorer clinical responses *(65)*. In another report, a weekly Oncaspar schedule was associated with a better complete remission rate (95%) in children with relapsed ALL (L-Asp non-naive), compared with children with the same characteristics but undergoing a biweekly schedule (82%). Among these patients, resistance to chemotherapy treatment was more frequently observed in association with significantly lower median plasma L-Asp activity levels and higher titers of anti-L-Asp antibodies *(78)*. Woo et al. *(64)* have recently reported the results of a study performed on 154 children to evaluate whether a possible relationship exists between overt hypersensitivity reaction or silent development of antibodies

and outcome. Most of the reacting patients were switched to the Erwinase preparation. No adverse prognostic impact was found for clinical or subclinical allergy to L-Asp.

The risk of developing an allergic reaction increases not only with the increasing number of L-Asp doses administered within the same treatment phase, but also when the drug is readministered after a break in treatment (e.g., in different chemotherapy phases within the same treatment protocol or in different protocols). In the frame of the ALL-BFM-90 study, medac L-Asp was given to all patients at conventional doses (10,000 IU/m^2 iv) during induction (×8) and reinduction (×4). During the consolidation phase, a randomized study was designed to answer the question of whether the addition of high-dose L-Asp (i.e., 25,000 IU/m^2 iv) given after each high-dose MTX cycle (overall four cycles) could reduce the incidence of relapses. This trial showed an increased percentage (i.e., 75%) of patients presenting with allergic reactions in the high-dose L-Asp treatment arm during the consolidation phase compared with children (34.5%) randomized not to receive high-dose L-Asp *(12)*.

Patients reacting to native products are usually switched to another non-cross-reactive product and are often able to complete the scheduled L-Asp treatment successfully *(11,12,29–34,44,45,51)*. Allergic reactions are rarely seen when the PEG-L-Asp product is used in frontline treatments *(51–55)*. In the case of a switch to the PEG product, patients are often able to receive the whole planned L-Asp treatment with no further signs of allergy *(79)*. It should be kept in mind, however, that in some patients previously either reacting or not reacting to native preparations, the absence of clinically detectable allergic reactions does not necessarily imply (owing to possible onset of silent inactivation) the achievement of therapeutic efficacy *(55)*. Because of this possibility, monitoring of L-Asp activity levels seems advisable.

It is very difficult to predict which patients will experience an allergic reaction to L-Asp administration. In some institutions a testing dose is performed before drug administration, but the benefit of this procedure is controversial, given that the dose test can itself induce patient sensitization *(29,32)*. Since the early 1970s, it has been reported that a markedly shortened L-Asp half-life was observed not only after, but also before, the onset of overt allergic reaction *(80)*. Conversely, it has been anecdotally reported that L-Asp activity levels may still be measurable after the occurrence of an allergic reaction *(45)*.

Anti-L-Asp antibody levels increase with repeated exposures to the drug, with higher levels found in patients who develop hypersensitivity reactions during further exposures *(62)*. The occurrence of high-titer anti-L-Asp antibodies may thus suggest not only an insufficient enzymatic activity (silent inactivation) but also a higher risk of allergic reaction to a specific L-Asp preparation during subsequent reexposures.

7.2.Protein Synthesis Inhibition

Once asparagine depletion occurs during L-Asp treatment, lymphoid blast cells suffer from impaired protein synthesis, ultimately leading to leukemic cell death. "Normal" cells, especially those constituting tissues with elevated metabolic turnover and having limited asparagine synthetase activity, may also be variously affected by the same mechanism of action; this provides the basis for different toxicity patterns observed during L-Asp treatment. Hypolipoproteinemia with elevation of triglycerides and cholesterol, hypoalbuminemia, and reduced synthesis of many hormones may also be detected with laboratory tests. These anomalies are rare and have only slight clinical relevance, and their occurrence does not necessitate specific treatment *(15,16)*.

7.3. Liver

The acknowledgment that L-Asp may be responsible for causing liver function anomalies is difficult to assess in the context of multidrug chemotherapy schedules. Elevated liver enzymes and bilirubin levels, as well as fatty degeneration of the liver, are generally reported as the most frequent liver anomalies *(15–17)*. Symptoms usually disappear completely after drug discontinuation, but liver dysfunction can also be severe, prolonged, or sometimes life-threatening *(81,82)*. Hepatic protein biosynthesis impairment also leads to coagulation disturbances with possible onset of thrombotic or hemorrhagic complications *(46,47)*. In retrospective nonrandomized comparisons, Erwinase has been reported to be less hepatotoxic than Crasnitin *(13)*. Different patterns of liver function anomalies have been reported for PEG-L-Asp products compared with those observed for the native forms *(79,83,84)*.

7.4. Central Nervous System

Because of its high molecular weight, L-Asp does not cross the blood-brain barrier. Neurologic disturbances such as drowsiness, lethargy, or confusion have therefore been attributed to the indirect action of the drug on the brain cells, through the release of high amounts of ammonia or aspartic or glutamic acid, or the decline in serum or CSF levels of asparagine or Gln *(72,85)*. Symptoms usually disappear completely after drug discontinuation. In some patients presenting with severe CNS disturbances, asparagine has been administered with some benefit *(81,86)*.

7.5. Coagulation

During L-Asp treatment, reduced biosynthesis of important clotting factors (antithrombin III, fibrinogen, plasminogen, protein C) may induce mild-to-severe coagulation derangement, which may result in hemorrhagic or thrombotic events in 1–5% of patients *(15–17)*. In some reports, the incidence of thrombotic events has been >10%, with a significantly increased risk for children with central venous lines and/or with a genetic predisposition to thrombosis *(87)*. The severity and incidence of hemostatic change may depend on the L-Asp product used or even the dosage adopted (when the same product is used) *(88,89)*. Concomitant use of corticosteroids, which may alter endothelium integrity, may represent an additional risk factor for development of coagulation disorders. Also, specific clinical conditions such as severe infection or chemotherapy-induced thrombocytopenia may increase the risk of thrombotic or bleeding incidents. A strict coagulation test monitoring is generally performed during L-Asp treatment. However, which supportive prophylactic procedures should be undertaken in the presence of coagulation alterations is a matter of debate. Several studies aimed at reducing the risk of thrombosis through supplementation of fresh frozen plasma or antithrombin III preparates have been reported *(90,91)*.

Whether any kind of supplementation is really capable of reducing the incidence of thrombotic episodes remains unclear. In fact, the relatively low incidence of clinically relevant thrombotic episodes makes it quite difficult to evaluate correctly whether a specific prophylactic treatment could help to decrease the incidence of thrombotic events. At present the first step should be the definition of patients at higher risk of developing thrombosis (e.g., those bearing genetically determined prothrom-botic risk factors or those with central venous lines or prolonged bed confinement or young female adolescents taking oral contraceptives) *(88,93)*.

7.6. Pancreas

A few days or weeks after L-Asp administration, acute pancreatitis may occur in 2–16% of cases *(93)*. However, clinically relevant symptoms (anorexia, nausea, vomiting, fever, epigastric pain) are only evident in a minority of patients; elevated serum or urinary amylase and abdominal ultrasound may be helpful for diagnosis. Less than 1% of patients treated with L-Asp are at risk of developing life-threatening hemorrhagic pancreatitis *(94)*. The mechanism of this complication is poorly understood. Treatment usually consists of supportive measures such as fasting and intravenous fluids. Somatostatin has been reported to be beneficial, given its pancreatic enzyme secretion suppression *(95)*. A pseudocyst may occur a few weeks or months after pancreatitis, and, in some cases, surgery is required for this complication *(96)*.

The incidence of pancreatitis in children with newly diagnosed ALL treated with Oncaspar has generally been reported to be similar or lower compared with that observed with native forms *(55,57,83)*. However, in a cohort of ALL patients who had already been exposed to native forms of L-Asp, an increased incidence of pancreatitis was reported after Oncaspar treatment *(97)*. L-Asp administration is generally discontinued in patients experiencing L-Asp-related pancreatitis, especially when severe symptoms have occurred; however, retreatment with Erwinase proved successful in an anecdotal report *(95)*.

Endocrine function may also be altered during L-Asp treatment. Diabetic ketoacidosis as well as nonketotic hyperglycemia due to suppression of insulin production is mainly observed when steroids are administered concomitantly with L-Asp. Since symptoms may be severe and persistent in some cases, checks on blood and urine glucose levels should be routinely carried out on patients undergoing L-Asp treatment so that substitutive insulin therapy can be started whenever it is deemed necessary *(15–17)*.

8. WHAT QUESTIONS SHOULD CLINICAL TRIALS ADDRESS OVER THE NEXT DECADE?

Extraordinary advances have been achieved over the last few decades in the field of pediatric hematology oncology. In the early years, chemotherapy schedules were mainly based on empiric use of antileukemic drugs. With time, better supportive therapeutic measures and an increased knowledge of the therapeutic and side effects of anticancer drugs have become available. Treatment optimization has brought about major benefits for patients in terms of quality of life and outcome. A certain percentage of patients, however, experience serious side effects and disease relapse. Although many factors can influence the onset of such complications, a better knowledge of the pharmacologic characteristics and biologic insights into the sensitivity and resistance mechanisms of L-Asp could help to avoid untoward side effects and prevent relapse by improving its therapeutic index. Studies of this type are therefore needed. In particular, dose finding studies with well-defined pharmacologic end points should be performed in future clinical trials to establish dosing guidelines and efficacy/toxicity profiles specific for each available L-Asp preparation.

In the near future, a better L-Asp treatment could be offered by human, recombinant genetically manufactured preparations of the enzyme. If such products lack immunogenic properties, the well-known allergenic effects of currently available L-Asp preparations (produced with foreign enzymes) could be overcome.

An even better use of L-Asp will be possible when dose adaptation is performed for every single patient, by monitoring specific pharmacologic end points. Timely dosage refinement, intervals adjustment, or product shifts based on findings of drug monitoring studies could theoretically minimize drug inefficiency, which is indirectly responsible for some treatment failures. A similar strategy has already proved effective in single-center trials, where it is easier to manage the multiple economic, organizational, and technical aspects *(98)*. Future prospective randomized clinical trials could thus evaluate whether this strategy is applicable and (cost)-effective in cooperative multicenter national or international trials.

Such trials could be performed more easily if laboratory tests for monitoring biologic correlates of L-Asp efficiency were less expensive and easier to perform. Efforts to achieve this goal are ongoing. Such tools will soon be available for application in clinical practice.

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Antileukemic Drugs

III

C

Antipurines in Childhood Acute Lymphoblastic Leukemia

29 Antipurines in Childhood Acute Lymphoblastic Leukemia

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CONTENTS

1. INTRODUCTION

6-Mercaptopurine (6MP), an antipurine, remains a mainstay of contemporary treatment of acute lymphoblastic leukemia (ALL) in infants, children, and adolescents. Its antileukemic activity was first reported in 1953—almost a half century ago. 6MP found a place in ALL therapy in the late 1960s, about 30 yr ago *(1,2)*. Since then, more novel, perhaps more interesting, and certainly more fashionable agents have garnered much attention and enthusiasm. *Tried and true 6MP differs from these exciting agents in that it has already contributed to the cure of literally tens of thousands of children with ALL.*

Despite long experience and dreary familiarity, important questions remain unanswered. The undeniable role of prolonged maintenance therapy in childhood ALL *(3)* eludes a fully satisfying understanding. Prolonged maintenance therapy has more effect on event-free survival (EFS) than on survival. The recent report of the Tokyo Children's Cancer Group finds that 1 yr of therapy including only 6 mo of maintenance therapy is sufficient for about 60% of children *(4)*. With abbreviated maintenance therapy, the usual EFS advantage for standard-risk patients *(5)* based on age and white blood cell count disappeared, but the clear survival advantage remained.

Invocation of an alternative immunosuppressive effect for maintenance therapy lacks corroboration in the apparent absence of a graft-versus-leukemia effect in the context of allogeneic bone marrow transplantation. Low-dose cytotoxic drugs certainly seem more likely to favor the emergence of resistant clones than to cure patients. However, thiopurines may accumulate gradually and disproportionately in rapidly dividing nucleated cells and may induce apoptosis when some threshold is reached. In theory, prolonged therapy may suppress the emergence of metachronous leukemia that masquerades as later relapse.

Recent trials have examined the dose, schedule, and route of administration of 6MP. In a number of recently completed and ongoing trials, 6MP is being compared with its analog, 6-thioguanine (6TG). Currently, a variety of nonmethylated and methylated thiopurine metabolites are being assayed, but their individual therapeutic roles remain to be defined *(6,7)*. Questions remain as to whether modified use of antipurines, important components of contemporary therapy, may further improve the outcome of therapy for childhood ALL and whether thoughtful application of the growing understanding of host polymorphisms, antipurine pharmacokinetics, and pharmacodynamics may lead to their more effective use.

2. MECHANISMS OF ACTION

In rapidly dividing cells, a number of critical enzymes in nucleotide metabolism and DNA synthesis may be upregulated and provide inviting therapeutic targets *(8)*. Despite the pedagogic imperative to link a single drug with a single, straightforward mechanism of action, drug effects are often complex and dose-dependent. The attribution of clinical benefit to a particular moiety or a particular mechanism usually

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remains speculative, no matter how viscerally satisfying the connection may be.

The antipurines are generally prodrugs and require conversion to nucleosides and nucleotides in order to exert their anticancer activity *(9)*. The nucleoside analogs include the arabinosyl adenines, thiopurines, and arabinosyl guanines. 6MP and 6TG, the most commonly employed antipurines in ALL therapy, are thiopurines and are discussed in more detail below.

2.1. Adenosine Arabinoside and Analogs

Adenosine arabinoside inhibits ribonucleotide reductase (RR), but its efficacy is limited by its rapid catalysis by adenosine deaminase. Fludarabine, cladribine, and clofarabine were developed to resist this deamination. All are self-potentiating; that is, they decrease intracellular levels of deoxynucleotides and thereby increase their own incorporation into DNA, which in turn impedes chain elongation in scheduled (S-phase associated) and unscheduled (DNA repair-associated) DNA synthesis. Other reported effects include inhibition of DNA and RNA primer formation and inhibition of mitochondrial DNA synthesis at lower concentrations and depletion of intracellular adenosine triphosphate and nicotinamide adenine dinucleotide at higher concentrations *(9)*.

Cladribine is also toxic to nondividing cells that are not dependent on ribonucleotide reductase *(9)*. Fludarabine may have a role in the treatment of acute myeoloblastic leukemia *(10–12)*. Fludarabine, cladribine, and pentostatin are active against adult low-grade lymphoma, in decreasing order of activity *(13)*.

2.2. Adenosine Deaminase Inhibitors

Pentostatin (deoxycoformycin), derived from *Streptomyces* species, also resembles adenosine and inhibits adenosine deaminase, leading to accumulation of toxic phosphorylated metabolites of deoxyadenosine. These metabolites, in turn, inhibit ribonucleotide reductase, a key enzyme in the synthesis of deoxyribonucleotides *(13)*. Hydroxyurea also inhibits ribonucleotide reductase.

2.3. Guanosine Arabinoside

2-Amino-9-β-D-arabinosyl-6-methoxy-9H-guanine (506U) is a prodrug of arabinosyl guanine (ara-G), an analog of deoxyguanosine. It has substantial activity against T-lineage ALL in children and adults *(14)*. This effect is mediated through accumulation of its toxic 5'-triphosphate, araGTP, which is resistant to degradation by purine nucleoside phosphorylase *(15)* and preferentially concentrated in T- lymphocytes and lymphoblasts compared with B-lineage *(16)*. 506U is under study in T-cell malignancies. Neurotoxicity in the form of coma and cerebellar toxicity has been dose-limiting *(14)*. Further trials to define the role of this antipurine in the treatment of childhood T-lineage ALL are under way.

2.4. Thiopurines

The thiopurine 6MP is a prodrug *(2,6,7)*. Therapeutic activity follows cellular entry by passive diffusion and activation by reaction with 1-pyrophosphoribosyl-ribose-5'-phosphate (PRPP) to form thioinosine monophosphate (TIMP), mediated by hyoxanthine-guanine phosphoribosyl tranferase (HGPRT). Inside the cell, nucleotides may be degraded back to nucleosides by cytoplasmic 5-nucleotidase and nonspecific phosphatases such as acid and alkaline phosphatase *(17)*. Uncharged nucleosides readily diffuse out through the cell membrane with the concentration gradient.

Many metabolites have biologic activity in vitro. TIMP blocks the first step of *de novo* purine biosynthesis via inhibition of PPRP amidotransferase (PRPP-AT). Cells in culture may be rescued from 6MP by 5-amino imidazol-4 carboxymide ribonucleotide, an early purine precursor *(9)*. TIMP also blocks the synthesis of adenine nucleotides by inhibiting the conversion of inosinic acid to adenylic acid by adenosylsuccinate synthase and the synthesis of guanine nucleotides by inhibiting the oxidation of inosine to xanthylic acid. Ultimately, intracellular pools of purine nucleotides may be depleted. A lesser amount of TIMP is converted to thioguanylic acid, which may be incorporated into both RNA and DNA in nucleated cells. In red blood cells, thioguanine nucleotides (TGNs) accumulate and are retained because of their charged phosphate moiety. Another metabolite, methyl mercaptopurine nucleotide, is formed by thiopurine methyl transferase (TPMT) and impedes *de novo* purine biosynthesis via inhibition of PRPP-AT. In the methyl-thiazol-tetrazolium (MTT) assay, a second methylated metabolite, methyl TIMP, is 20-fold more cytotoxic than methyl meraptopurine nucleotide *(18)*.

The thiopurine 6TG is the 6-thiol derivative of guanine. It is about 10-fold more potent than 6MP against leukemic cell lines *(19)* and leukemic lymphoblasts obtained from patients with ALL *(17)*. Red cell accumulation of TGNs is about fivefold higher with 6TG than with isotoxic doses of 6MP *(20)*, suggesting that TGNs are not the only determinant of myelosuppression. 6TG probably provides more extensive incorporation into DNA and RNA than does 6MP. Following activation via HGPRT, di- and triphosphates are readily formed and incorporated into RNA and into DNA after conversion to thioguanine deoxyribonucleoside (TGdR) via DNA polymerase. Replacement of 0.1% of guanine in RNA or of 0.4% in DNA markedly diminishes cell viability *(7)*. After integration into DNA, thioguanine is methylated by S-adenosylmethionine to S^6-methylthioguanine.

The postreplicative DNA mismatch repair system mediates cell death. During DNA replication, the S^6-methylthioguanine directs incorporation of either thymine or cytosine. The postreplicative DNA mismatch repair system recognizes resulting S^6-methylthioguanine-thymine base pairs as mismatches and initiates apoptosis, analogous to the recognition of O^6-methylthioguanine-thymine base pairs induced by nitrosoureas *(21,22)*. Hence 6TG and *N*-methyl-*N*-nitrosourea cytotoxicity appear to share dependence on postreplicative DNA mismatch repair. Again, methylated metabolites may have some role. A methylated metabolite, S-methyl TGN, inhibits PRPP-AT but is about 12-fold less active than methyl mercaptopurine ribonucleotide *(23)*. 6TG does not appear to impede *de novo* purine biosynthesis or to deplete intracellular purine nucleotide pools *(9)*.

Curiously, sensitivity to 6TG—not 6MP—has been reported in a number of HGPRT-deficient cell lines via incorporation into the anticodon of specific tRNA isoacceptors in place of the unusual base queuine found only in the wobble position of isoacceptors for asparagine, aspartic acid, histidine, and tyrosine *(24,25)*. On the other hand, 6MP may be

active against some 6TG-resistant cell lines. The methylated 6MP metabolite, methyl TIMP, was found to inhibit *de novo* purine synthesis via PRPP-AT *(7)* and to exhibit cytotoxicity aginast a 6TG-resistant MOLT-4 subline as well as 6TG-sensitive cell lines *(18)*.

In vitro resistance to thiopurine at diagnosis, as assessed by the MTT assay, correlated with an increased risk of relapse *(17,26,27)*. The ratios of the median lethal concentration (LC_{50}) values for 6TG and 6MP obtained at presentation and relapse were 1.3 and 1.5. In contrast, the ratio for prednisone was 357 *(28)*. In experimental tumor systems, resistance is associated with loss of HGPRT activity. Limited human data indicate that resistance is associated with failure to accumulate intracellular ribonucleotides. In addition, high levels of ecto-5'nucleotidase have been associated with a poor outcome for CD10-positive ALL *(17)*. Ecto-5' -nucleotidase on the cell membrane degrades extracellular nucleotides to nucleosides, which may readily diffuse into a cell and contribute to purine salvage pathways.

In summary, three potential mechanisms of activity have been invoked, namely, inhibition of *de novo* purine synthesis by both TIMP and methylated metabolites, incorporation into DNA, and incorporation into specific tRNAs. The relative clinical importance of inhibition of *de novo* purine synthesis by nonmethylated and/or methylated moieties vs incorporation into DNA and/or RNA remains to be determined.

De novo purine synthesis is increased in cancer cells compared with normal cells. Purine synthesis via salvage pathways may make *de novo* synthesis superfluous. However, the role of *de novo* synthesis may be underestimated in tissue culture, where purine is available from hypoxanthine in the medium and from dead cells via salvage pathways *(23)*.

Some argue that the incorporation of thiopurines into DNA and RNA, and not the inhibition of purine synthesis, is the principal mechanism of cytotoxicity *(7,23)*. Methylated metabolites are considered inactive, and TPMT is viewed as a catabolic enzyme. In support of this notion, Keuzenkamp-Jansen et al. *(29)* found that biweekly intravenous 6MP failed to deplete levels of ribonucleotides in peripheral blood mononuclear cells. Others argue for a larger role of the inhibition of purine synthesis. They point out that the levels of thioguanine nucleotides tolerated in patients on 6MP with TPMT deficiency or on 6TG are much higher than those tolerated in patients on 6MP with normal TPMT and therefore that methylated metabolites must be cytotoxic *(18)*. At the other extreme, Pizzorno and coworkers *(9)* conclude that data are lacking to establish any significance of DNA/RNA incorporation for cytotoxicity or anticancer. Both mechanisms may be involved. Any inhibition of *de novo* purine synthesis may enhance incorporation of TGNs into DNA *(30)*.

3. PHARMACOKINETICS

The bioavailability of oral 6MP is low and highly variable among patients and averages around 16% *(31)*. Bioavailability may be greater at lower doses of 100–200 mg/m^2 and relatively lesser at higher doses of approx 500 mg/m^2 *(6,32)*. Oral 6MP is vulnerable to conversion to thiouric acid by xanthine oxidase (XO) in the gut or on first passage through the gut and liver. Allopurinol, an XO inhibitor, increases 6MP bioavailability after oral administration but not after intravenous administration *(33)*. The population variability of XO remains to be explored.

The basis for the high degree of population variability in the bioavailability of 6MP remains to be elucidated. Diet may provide part of the answer. Milk has XO activity *(34)*. Ingesting 6MP with milk might degrade the drug before it reaches the systemic circulation.

Balis and coworkers *(35)* studied 6MP pharmacokinetics in 89 children. Following an oral dose of 75 mg/m^2, the area under the plasma concentration-time curve (AUC) varied 70-fold, from 0.11 to 8 μmol × h/L. Median erythrocyte TGN levels ranged from 0 to 10 pmol/g hemoglobin. Dose lacked any correlation with either the AUC or the erythrocyte TGN level. The median plasma elimination half-life was 1.5 h *(31)*. Intrapatient variability was substantial. The cerebrospinal fluid (CSF) penetration of 6MP was 20% *(36)*, and cytotoxic CSF levels are not likely after oral dosing.

4. 6MP IN CHILDHOOD ALL

As a single agent, 6MP induces remission in between 27 and 64% of children with ALL, compared with 40–85% for vincristine, and 22–40% for methotrexate. When therapy is halted after remission induction, the median duration of remission is no more than several months *(37)*. Patients in remission are believed to harbor a small burden of residual leukemia cells with a high growth fraction and thereby an increased susceptibility to S-phase-specific antimetabolite therapy.

Childhood ALL is unique among human malignancies in the proven value of prolonged low-dose maintenance therapy *(3)*. Among patients in remission after vincristine and prednisone induction, daily oral 6MP increases the median duration of remission from 2 to 7 mo. The combination of daily oral 6MP and once or twice weekly oral methotrexate is superior to either cyclic (drugs rotated at fixed time intervals) or sequential (second drug introduced after disease progression on first drug) administration of the same agents *(37,38)*.

In the laboratory, the combination of 6MP and methotrexate is synergistic. Methotrexate inhibits *de novo* purine synthesis, resulting in increased levels of intracellular PRPP and decreased intracellular pools of purine nucleotides. Higher levels of PRPP enhance phosphorylation and intracellular accumulation of thiopurines. Smaller pools of competing purines result in more extensive DNA and RNA incorporation of phosphorylated thiopurines *(2)*.

Most contemporary treatment programs for childhood ALL administer daily oral 6MP at doses from 50 to 75 mg/m^2/d in prolonged courses to patients in remission after more intensive induction and postinduction intensification therapy. The UKALL IV and V studies in the United Kingdom both found that intermittent maintenance treatment was inferior to continuous therapy and that nearly continuous therapy, 3 of every 4 wk, had intermediate efficacy *(39)*. The quite successful Dana Farber Cancer Institute protocols 81-01 and 85-01 administer 6MP during 3 of every 4 wk *(40,41)*.

Other schedules have been examined. A Children's Cancer Group (CCG) trial found that standard daily oral 6MP and weekly oral methothrexate with monthly vincristine and pred-

nisone pulses was more effective than a more toxic, intermittent regimen consisting of alternating pulses of prednisone, vincristine, methotrexate, and 6MP (POMP) with prednisone, vincristine, cytosine arabinoside, and Adriamycin (POCA) every 4 wk *(42)*. On the other hand, Koizumi and colleagues *(43)* showed an advantage for an intermittent maintenance program of 6MP 175 mg/m^2/d orally × 5 d alternating each 2 wk with methotrexate 225 mg/m^2 intravenously over a conventional daily oral 6MP and weekly oral methotrexate regimen.

Pinkel and coworkers *(44)* compared full- and half-dose maintenance therapy with 6MP and methotrexate and found the full dose superior. Further attempts to intensify maintenance by adding additional agents (cyclophosphamide and/or cytosine arabinoside) to the basic daily oral 6MP and weekly oral methotrexate maintenance scheme diminished administration of 6MP and methotrexate, increased toxicity, and decreased EFS *(45)*. On the other hand, addition of vincristine and prednisone pulses has shown benefit in one trial *(46)*. Patients received no other postinduction intensification

Current 1999 CCG dose modification guidelines for maintenance regimens call for dose escalation of methotrexate and thiopurine by 25%, alternately, and no more frequently than each 2 mo, for an absolute neutrophil count >1500/μL and platelet count >75,000/μL on three successive monthly complete blood counts. The dose of 6MP is halved for an absolute neutrophil count between 500 and 750/μL or a platelet count between 50,000 and 75,000/μL and omitted for an absolute neutrophil count <500/μL or a platelet count <50,000/μL. Once withheld, 6MP is restarted at half-dose, once the peripheral blood counts have recovered with an absolute neutrophil count >1000/μL and a platelet count >100,000/μL. Thiopurine is withheld for elevation of conjugated bilirubin or for hepatomegaly and not simply for isolated elevation of transaminases or of unconjugated bilirubin. These recommendations are somewhat arbitrary, based neither on sound pharmacologic rationale nor on data from clinical trials.

Substantial data exist to show that lesser exposure to 6MP decreases EFS. Over the course of treatment, children generally receive between 700 and 1000 d of treatment with 6MP, mostly in maintenance or continuation phases following some earlier period of more intensive induction and postinduction intensification therapy. Decreased dose intensity *(44,47,48)* and/or an abbreviated duration of treatment *(3)* leads to decreased EFS rates. Bioavailability varies widely among individuals after oral administration *(31,35)*, and those with lesser intracellular accumulation of active metabolites have had a worse outcome in some studies *(49,50)*. Treatment adherence by patients and physicians *(51–53)* and gene polymorphisms *(54–59)* may play critical roles in treatment outcome.

Koren and coworkers *(60)* studied 23 patients and found an association between the plasma AUC following a single oral dose during maintenance therapy and outcome. Similarly, Peterson and coworkers *(61)* linked a high AUC to myelotoxicity and freedom from relapse. Such a claim required normalization to an inverse function of apparent clearance, and thus its validity has been challenged *(62)*. Other studies, in larger numbers of patients, have found no simple and straightforward association between plasma pharmacokinetics and outcome *(35)*.

Lilleyman and Lennard *(50)* studied 172 patients on oral 6MP therapy and found a median erythrocyte TGN level of 284 pmol/8 × 10^8 erythrocytes. Patients with values below the median were 2.5 times more likely to relapse. Erythrocyte TGN levels correlated with neutropenia. Similarly, Bostrom and Erdmann *(49)* found an excess of relapses among children with erythrocyte TGN levels below the group median. However, a substantial overlap in erythrocyte TGN concentrations was found between patients with relapse and those in continued remission.

Relling and coworkers *(48)* linked outcome with 6MP dose intensity in multivariate analyses. Underexposure to 6MP may result from chronic underdosing and/or from prolonged interruptions in therapy. The St. Jude researchers found that neutropenia and interruptions in therapy were more damaging to dose intensity and outcome than was chronic underdosing. Contrary to Lilleyman and Lennard's seminal study *(50)*, they found no simple link between erythrocyte TGN and outcome. However, in Relling's study the median TGN level was 401 pmol/8 × 10^8 erythrocytes, substantially higher than the 284/8 × 10^8 erythrocytes in Lilleyman and Kennard's report. van Eys and colleagues *(63)* sought unsuccessfully to improve outcome by pushing for maximal 6MP dosing.

In 1984, Rivard and coworkers *(64)* reported that children who received 6MP in the evening were less likely to relapse than children who took 6MP in the morning. Koren and coworkers *(65)* found a greater than 2-fold longer half-life and 1.5-fold larger AUC with evening administration. Clinically, evening administration resulted in greater myelosuppression. However, repeated studies on the same patient showed a high degree of intrapatient variability, which argues for caution in interpreting conclusions based on data from a small number of samples derived from a small number of patients, no matter pleasing the conclusions *(35)*. Schmiegelow and coworkers *(66)* also found an improved outcome in a self-selected population choosing evening administration, 82% vs 57% (p = 0.0002), although the levels of erythrocyte TGN were similar. Bedtime administration has been largely adapted.

Children with ALL are generally treated for 2–3 yr *(3)*. One study showed no advantage for 5 yr of therapy over 3 yr of therapy *(67)*. A second trial showed that 18 mo of therapy was inferior to 2 yr *(68)*. Several randomized trials compared 2 and 3 yr and found a moderate EFS advantage for 3 yr—slightly greater in males and slightly lesser in females. No survival advantage was proved, leaving the issue unresolved *(3)*. Many of these trials were conducted before contemporary advances in therapy. A smaller fraction of patients remained in remission at 2 yr and thus eligible for randomization for duration of therapy than is currently achieved. We now have a greater percentage of patients in remission at 2 yr than formerly. The current CCG practice is to treat girls for 2 yr and boys for 3 yr from the start of interim maintenance therapy or 26–27 mo and 38–39 mo in total, respectively.

6MP and its analog 6TG have been administered intravenously. Zimm and coworkers *(31,36)* found a sixfold increased plasma AUC and more predictable exposure with intravenous as opposed to oral administration. Sunderland and coworkers *(69)* examined erythrocyte accumulation of TGNs after intra-

venous 6MP. Patients received 6MP at 1 g/m^2 over 20 h and reached peak levels of 351 pmol/8 × 10^8 erythrocytes by 36 h after the start of the infusion, with trough levels of 53 pmol/8 × 10^8 erythrocyte at 7 d, no better than those achieved with oral 6MP. Steady state was not reached. Among 40 patients with relapsed ALL, one complete response and one partial response were obtained after treatment with 6MP 2.4 g/m^2, given by 48-h infusion *(70)*. Outstanding, although preliminary, results in a Pediatric Oncology Group (POG) pilot trial *(71)* led to a number of randomized trials, none of which has yet to show an advantage for parenteral 6MP *(72–75)*. In fact, one trial showed a worse EFS when the usual oral 6MP was supplemented with additional parenteral 6MP *(72)*. A second trial found inferior survival but a similar EFS when oral 6MP was replaced with weekly parenteral 6MP in mo 2–4 after remission induction *(75)*.

Explanations for the above findings are difficult to find. 6MP enters cells by passive diffusion, and high plasma levels should lead to high intracellular levels. However, a larger percentage of drug appears unchanged in the urine after intravenous as opposed to oral administration *(36)*. Bioactivation by HGPRT may be rate-limiting. A high plasma level of 6MP may paradoxically decrease intracellular TGN levels and cytotoxicity *(76)*. Very high levels of 6MP, probably higher than those attainable clinically, may result in desulfuration and less DNA incorporation of TGN than obtained at lower concentrations *(77)*.

5. 6-MERCAPTOPURINE VS 6-THIOGUANINE

6MP was licensed before 6TG and has been used in most trials in childhood ALL. Both drugs are readily converted to thiouric acid, and both may be methylated by TPMT.

Adamson and coworkers *(19)* found that 6TG was 1 log more active in vitro than 6MP. In patients, isotoxic doses of 6TG provide five-fold higher levels of intracellular TGNs *(20,78–80)*. A CCG/Pediatric Oncology Branch pharmacology trial found potentially therapeutic plasma and CSF levels with intravenous 6MP and 6TG and oral 6TG. With oral 6MP, levels were apparently subtherapeutic *(83)*. However, the relation between in vitro and in vivo activity is uncertain. Evans and Relling *(23)* caution that in vitro conditions may lessen the impact of any inhibition of *de novo* purine synthesis. Levels of erythrocyte TGNs correlate with myelosuppression after treatment with 6MP but not after 6TG *(20)*. Clinically, 6TG induces thrombocytopenia and mild, self-limited cases of hepatic venoocclusive disease, not seen with 6MP *(84)*.

Isotoxic comparisons of 6MP and 6TG are being conducted by several groups. Preliminary results from the German CoALL Group show no advantage for 6TG *(85)*. Larger U.S. and U.K. studies are pending. Methylated metabolites may contribute to efficacy and toxicity.

6. ADHERENCE TO MAINTENANCE THERAPY

Prolonged maintenance therapy presents challenges in adherence or compliance for both families and physicians. Appropriate dose modifications over a long course of therapy in the face of toxicity present a constant challenge to physicians. Davies and coworkers *(51)* monitored erythrocyte TGN levels in 35 children, observing levels that varied by twofold or more among multiple determinations in 6 children and suggest poor compliance as the cause. Lau and coworkers days *(53)* monitored adherence in 24 patients by means of a Medication Event Monitoring System pill container. Over a 15–94 d interval (mean, 44 d), 8 of 24 patients missed >10% of days of treatment, and 4 patients missed >20% of days. Hale and coworkers *(47)* compared the 6MP prescribed for 30 children diagnosed before 1980 and 50 children diagnosed after 1980 in the United Kingdom. Before and after 1980, the target dose of 6MP remained the same, but after 1980, modifications of dose were made more stringent in an attempt to increase the amount of 6MP administered. The median total dose of 6MP increased by 22%, of which less than half could be explained by explicit protocol changes. EFS improved by 18%.

7. GENE POLYMORPHISMS

Although much attention has focused on the *TPMT* gene *(54,57,58,81)*, the biologic fate of 6MP and 6TG depends on several genes, namely, *XO*, *HGPRT*, cytoplasmic 5'-nucleotidase, and nonspecific intracellular phosphatases such as acid and alkaline phosphatase. *XO* is responsible for inactivation of 6MP in the gut or upon first passage through the liver. *HGPRT* is responsible for the bioactivation of both 6MP and 6TG. *TPMT* is responsible for inactivation or, on the other hand, the synthesis of methylated moieties, namely, methyl mercaptopurine and methyl TIMP with important cytotoxicity. Cytoplasmic 5'-nucleotidase and nonspecific intracellular phosphatases convert charged nucleotides, to uncharged nucleosides which may then diffuse out of cells.

A genetic polymorphism exists, with 0.3% of individuals having undetectable levels of *TPMT* and 11% having intermediate levels. Erythrocyte levels reflect levels in lymphocytes *(55,82)*. Levels of activity may be genetically determined but may increase during treatment. In 1987, Lennard and coworkers *(83)* reported a link between erythrocyte levels of TGN and TPMT activity. Three adults lacking activity had severe leukopenia upon treatment. Lennard and others *(81,84,85)* have subsequently found higher erythrocyte TGN levels and more severe toxicity in a number of patients with homozygous loss of TPMT activity. Relling and coworkers *(48)*found that patients homozygous for normal *TPMT* required dose reductions in 7% of cases; those heterozygous for mutant *TPMT*, 35%; and those homozygous for mutant *TPMT*, 100%. Of patients requiring dose reduction, only about 1 in 30 will be homozygous for mutant *TPMT*. McBride and coworkers *(86)* reported severe myelosuppression in a patient with homozygous loss of TPMT activity with 6TG therapy. Alves and coworkers *(87)* found severe myelosuppression in four patients who were reported to be heterozygous for *TMPT*3A*. McLeod and coworkers *(81)* found no increased toxicity in 16 heterozygous patients. Relling and coworkers *(88)* found an increased incidence of brain tumors among irradiated patients with intermediate TPMT deficiency.

Contrary to much that has been published, Pettersson and coworkers *(18)* found no association between TPMT activity and erythrocyte TGN levels. The methylated metabolite methyl TIMP, which has been shown to inhibit PRPP-AT

and *de novo* purine synthesis, showed marked cytotoxicity in vitro. Patients with TPMT deficiency on reduced 6MP dosage schedules tolerate much higher levels of TGN than do patients with normal TPMT . Isotoxic doses of 6TG provide levels of erythrocyte TGN fivefold higher than 6-MP, proving that erythrocyte TGN cannot be the only determinate of cytotoxicity.

Genes other than *TPMT* may have important polymorphisms. *HGPRT* resides on the X-chromosome. Digestion with the restriction endonuclease *Bam*HI reveals a three-allele restriction fragment length polymorphism in the *HGPRT* locus. Allele frequencies in a Caucasian population were 0.77, 0.16, and 0.07 *(89)*. The clinical significance of these findings is unknown.

8. DRUG INTERACTIONS

The sequence-dependent interactions of antipurines with methotrexate *(90–93)*, with cytosine arabinoside (ara-C) *(94–96)*, and with DNA-damaging agents that induce unscheduled DNA synthesis during DNA repair *(97)* may provide further therapeutic opportunities. Methotrexate expands PRPP pools and increases activation of 6MP and 6TG. The interactions between methotrexate and 6MP have already been discussed. Pretreatment with methotrexate enhances 6TG cytotoxicity. In L1210 cells, 3-h pretreatment with methotrexate decreased the LD_{90} from 800 pM for simultaneous exposure to 0.9 pM. Incorporation of 6TG into RNA was increased more than threefold, whereas incorporation into DNA was decreased fourfold *(98)*. Pretreatment with antipurines increases the uptake of ara-C and formation of ara-CTP. Sequence-dependent synergism has been described for fludarabine and ara-C *(99)*, 6MP and ara-C *(100)*, and 6TG and ara-C *(101)*. Pretreatment with an alkylator induces sublethal DNA damage *(102)*. DNA repair requires unscheduled (i.e., cell cycle-independent) DNA synthesis, which in turn, enhances the cytotoxicity of antimetabolites such as fludarabine *(97)*.

9. THE FUTURE

Thiopurines play a major role in the treatment of childhood ALL. Although parenteral 6MP has not been a clear success, thorough study of metabolite data may provide insight into the reasons for its unexpected failure and open new avenues of investigation. Critical clinical comparisons of 6MP and 6TG are under way, but the jury is still out. Renewed attention has been focused on the potential importance of methylated metabolites. Definition of clinical contributions of the various nonmethylated and methylated mercaptopurine and thioguanine metabolites may suggest strategies to achieve an optimal arrray of active metabolites with individualized dosing *(103)* of some combination of 6MP and 6TG *(23)*. Examination of host factors should go beyond *TPMT* to include *XO* and *HGPRT* and other genetic polymorphisms. Microarray technology provides access to much of the underlying complexity. However, gene expression may vary among tissues and over time. Gene expression may differ from gene function. Patient and physician adherence to treatment protocols should not be overlooked as we seek to enhance drug accumulation and improve outcome.

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30 Antipurines

LYNNE LENNARD AND JOHN S. LILLEYMAN

CONTENTS

1. INTRODUCTION

The thiopurines mercaptopurine and (to a lesser extent) thioguanine form an essential component of so-called maintenance therapy of childhood acute lymphoblastic leukemia (ALL). The drugs were developed by Hitchings and Elion nearly 50 yr ago *(1)*, and mercaptopurine was first used clinically in 1953 *(2)*. Over the intervening years, it has become apparent that both drugs have considerable activity in many types of leukemia; as knowledge of their pharmacology continues to accumulate, there is renewed interest in their role in maintenance treatment of common childhood ALL. To understand why, it is necessary first to have an appreciation of the evolution and importance of maintenance therapy—what it is and how it works.

1.1. Maintenance Therapy and the Role of Thiopurines

The concept of maintenance therapy predates attempts to cure childhood ALL. The term was originally used for early palliative regimens in which drugs were given singly, either in cycles or sequentially, and the concept of remission being "maintained" was defined clinically, as the course of the disease was punctuated by repeated episodes of disease recrudescence until refractory progression occurred *(3)*.

At about the same time, however, the concept of potentially curative "total" therapy was being developed by Pinkel and colleagues *(4)*. They planned an all-out onslaught on the disease consisting of the four phases of remission induction, consolidation, central nervous system (CNS) therapy, and maintenance. The rationale for the 2–3 yr maintenance phase of oral antimetabolite chemotherapy was based on the analogy of tuberculosis, in which prolonged treatment is necessary for cure. It was also thought that the generation times of the tubercle bacillus and the leukemic cell were similar *(4)*.

Whatever the reasons behind the evolution of maintenance therapy, there is little doubt that extended treatment of the type serendipitously designed by the St. Jude team is necessary for the successful eradication of at least some types of childhood ALL. The giving or not of maintenance has rarely been the subject of clinical trials, but 30 yr ago a study in which children were randomly given twice weekly methotrexate (30 mg/m^2) or no further therapy after 5 mo of standard induction and consolidation showed a dramatic difference, with only 2 of the 18 untreated children remaining in remission after a year, compared with 37 of the 52 given methotrexate *(5)*. Although this trial is unique, there is plenty of other evidence, albeit circumstantial, to suggest that maintenance is effective even with more modern protocols *(6)*, and it is true to say that so far no successful schedule has been described without an extended phase of continuing chemotherapy, usually antimetabolite-based, usually including daily oral thiopurines, and usually lasting long enough for the total treatment time to cover 2–3 yr.

Oddly, childhood ALL is the only human cancer for which this approach is effective. The strategy flies in the face of basic oncologic principles, and as the late Professor Tim McElwain once famously pointed out, the worst way to treat cancer is to dribble in little bits of chemotherapy over a long period. So what is maintenance treatment in ALL actually doing? Killing leukemic cells continuously? This seems improbable. Patients with detectable minimal residual disease (MRD) after 3–5 mo of treatment are highly likely to relapse *(7–9)*, so the chemical debulking of the disease has to be to the point of less than one marrow cell in a million before maintenance can prevent treatment failure. (The suggestion that MRD-negative children do not need maintenance is illogical unless it is doing nothing for

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any patients, rather, the MRD-positive ones need something else. So what is going on?

The common type of childhood ALL is a tumor of early B-cells. Their development is arrested at the stage of initial immunoglobulin heavy-chain gene rearrangement, a spontaneous and random process that, in normal cells and if it happens to produce a potentially functional allele, leads on to survival to the next stage, light-chain gene rearrangement. Functionless heavy-chain rearrangement normally leads to cell death through apoptosis. Unsuccessful light-chain rearrangement also leads to cell death, so bone marrow B-cell production is a fairly inefficient process with only a small proportion of the precursors surviving.

Gale and Butturini *(6)* hypothesize that maintenance-sensitive ALL is epitomized by cells with unsuccessful immunoglobulin gene rearrangements that nevertheless survive (due to other genetic mutations) to produce the original malignant clone. Such cells, if reduced to very small numbers and suppressed for long enough, might eventually succumb to the apoptotic process that should have prevented their survival in the first place. Although this does not completely explain the strange kinetics of common childhood ALL, particularly the occasional long latency to relapse after withdrawal of therapy *(10)*, it does support the notion that the disease requires a prolonged period of therapy designed to prevent the growth of cells rather than reduce their numbers.

The therapeutic effect of maintenance is therefore best regarded as immunosuppressive rather than antineoplastic. To be successful it has to be sustained and continuous. Children who receive low drug doses and intermittent maintenance and who have long gaps in therapy or no episodes of myelosuppression are more likely to relapse. Conversely, those who receive higher drug doses, continuous therapy, and occasional episodes of neutropenia fare better *(4,11–14)*.

Efforts to stimulate the immune system rather than suppress it as a means of eradicating residual ALL have proved spectacularly unsuccessful. BCG vaccine was used as an alternative to orthodox maintenance in the randomized trial referred to above, which also had an arm offering no further treatment. Active immunotherapy proved greatly inferior to methotrexate and equivalent to no treatment *(5)*.

So, at least for some types of ALL, immunosuppressive maintenance is important *(15)*, and historically the two drugs most pivotal to its success have been undoubtedly 6-mercaptopurine and methotrexate. Of the two, mercaptopurine is traditionally the major component; as an understanding of its pharmacokinetics grows, so does the realization that inter- and intrapatient variability in this respect are potentially clinically important.

1.2. What Do Thiopurines Do During Maintenance Therapy?

The quick answer is that nobody knows, but it is possible to speculate (*see* Section 2.2.). As indicated above, it is unlikely that there is a continuous daily process of malignant cell killing for 1–2 yr. Children who are destined to be long-term survivors must have very few cells left after a full induction program and one or more blocks of consolidation treatment, with the numbers of residual blasts being below the detection limits of current methods of MRD detection. Such infrequent cells will probably not be cycling but will be in an extended G0 phase. Resting cells that are out of the mitotic cycle cannot be killed by thiopurines. They will, however, accumulate potentially toxic metabolites, which might eventually lead to their destruction in one of two ways. Either the intracellular metabolites themselves will suppress recruitment to cycle, so that the cell eventually undergoes spontaneous apoptosis as an unsuccessful B-precursor (the Gale hypothesis) *(6)* or, equally possibly, the cell is culled when it reenters the mitotic cycle *(16)* (for whatever reason) by incorporating the already present abnormal nucleotides during DNA synthesis. Either way, very low frequency cells can be picked off one by one over an extended period; however, for the strategy to be successful, the intracellular concentrations of drug-derived metabolites must probably be sustained at high concentration over long periods.

Achieving such a concentration in these low-frequency target cells therefore requires continuous thiopurine therapy at the highest tolerable doses, since on drug withdrawal the cells will eliminate the metabolites over time and then become able to survive recruitment to cycle. This could explain why intermittent therapy is less successful than continuous therapy *(12)*, why higher doses are more effective than lower doses *(4,14)*, and why the absence of evidence of occasional myelosuppression is a bad omen *(11,13)*.

2. METABOLISM OF THIOPURINES

Mercaptopurine and thioguanine (2-amino-mercaptopurine) have no intrinsic anticancer activity; they are prodrugs that undergo extensive intestinal and hepatic metabolism. Biotransformation occurs via three competing pathways: oxidation, methylation, and nucleotide metabolite formation (Fig. 1). In the liver and intestinal epithelium all three pathways compete for the available thiopurine substrate, but in many tissues, including blood cells, only parts of these metabolic routes will be active.

2.1. Metabolic Pathways

2.1.1. Oxidation

Oxidation of mercaptopurine, catalyzed by xanthine oxidase, is a catabolic route leading to the formation of thiouric acid *(17)*. Thioguanine is oxidized by aldehyde oxidase to produce 8-hydroxy-thioguanine *(18)*. Thioguanine functions as a xanthine oxidase substrate only after prior deamination by guanase *(19)*. The thioxanthine formed is then oxidized via xanthine oxidase to thiouric acid. The catabolism of mercaptopurine to thiouric acid occurs via the intermediate 8-hydroxy-mercaptopurine and to a lesser extent via thioxanthine *(20)*. Xanthine oxidase is present at high specific activities in the intestinal mucosa and liver but at low activities in other tissues *(21)*. Functional xanthine oxidase activity is absent in circulating blood cells. Population studies have reported a 4- to 10-fold interindividual variation in the liver enzyme activity *(17,22)*.

2.1.2. Methylation

S-Methylation of mercaptopurine catalyzed by thiopurine methyltransferase (TPMT) is a major metabolic route. Both mercaptopurine and thioguanine serve as substrates for TPMT

S-methylation (K_m= 383 and 557 μ*M*, respectively), but the methylmercaptopurine produced from mercaptopurine inhibits its human kidney TPMT activity (K_i of 560 μ*M*). Mercaptopurine nucleotide (thioinosine monophosphate) is also a good substrate for TPMT, some 18-fold better than the thioguanine nucleotides (TGNs), which function as poor substrates *(23)*. 8-Hydroxymercaptopurine is an excellent substrate (K_m = 96 μ*M*), but both thioxanthine and thiouric acid are potent inhibitors. Thiouric acid is the end product of xanthine oxidase-catalyzed mercaptopurine oxidation *(23)*, raising the possibility of a major interaction between these two primary pathways of mercaptopurine metabolism.

TPMT activity in the red blood cell and other human tissues, including the liver, is under the control of a common genetic polymorphism *(24,25)*. The frequency distribution of TPMT activity in Caucasian populations is trimodal: approx 89% of the population have high enzyme activity and are homozygous for the wild-type allele (*TPMT*^{H}), 11% inherit intermediate levels of enzyme activity with one wild-type and one variant allele (heterozygous *TPMT*^{H}/*TPMT*^{L}), and 1 in 300 subjects have no functional activity (two variant alleles, homozygous *TPMT*^{L}). A number of variant TPMT alleles have now been described *(26–28)*, and one can expect more to be documented in the near future.

2.1.3. Nucleotide Formation

Intracellular activation of mercaptopurine, catalyzed by the ubiquitous enzyme hypoxanthine phosphoribosyltransferase (HPRT), leads to the formation of a variety of active nucleotide metabolites. The initial mercaptopurine nucleotide is oxidized to thioxanthine nucleotide (thioxanthosine monophosphate) and then converted to a thioguanine nucleotide (TGN; thioguanosine monophosphate). Thioguanine is converted directly into a thioguanine nucleotide via HPRT (Fig. 1). Mercaptopurine and thioguanine are very good substrates for HPRT, but methylmercaptopurine is a very poor substrate; the methylthio group hinders binding to the HPRT active site *(29,30)*. It is drug-derived TGN that is eventually incorporated into DNA as a false base *(31,32)*.

There are two types of pathways leading to nucleotides, the *de novo* and salvage pathways. *De novo* synthesis begins with metabolic precursors, whereas salvage recycles the free bases and nucleosides released from nucleic acid breakdown. *De novo* purine synthesis occurs in lymphocytes and rapidly proliferating tissues, including lymphoblasts. In all other tissues, including neutrophils and macrophages, the HPRT purine salvage pathway predominates, and in mature red blood cells it is the only route of nucleotide formation.

2.2. How Thiopurine Metabolites Exert Their Effects

Thiopurine drug activation occurs intracellularly, and several active thionucleotide metabolites are formed. These thiopurine nucleotides compete with their endogenous counterparts in many biochemical pathways.

Nucleotides play a variety of important roles in all cells. They are precursors to DNA and RNA, they are essential carriers of energy [e.g., ATP and GTP), they are components of essential biochemical cofactors [e.g., nicotine adenosine dinucleotide (NAD) and *s*-adenosyl methionine], and some,

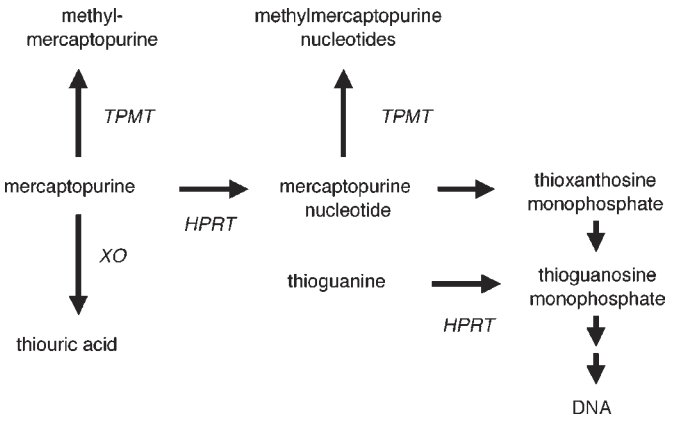

Fig. 1. Thiopurine metabolism. The initial metabolism of mercaptopurine proceeds along three competing routes, catalyzed by thiopurine methyltransferase (TPMT), xanthine oxidase (XO), and hypoxanthine phosphoribosyltransferase (HPRT). HPRT produces mercaptopurine nucleotide (thioinosine monophosphate), which is then oxidized to thioxanthine nucleotide (thioxanthosine monophosphate) by inosine monophosphate dehydrogenase and converted to a thioguanine nucleotide (thioguanosine monophosphate) by guanylate synthetase.

such as cyclic AMP and cyclic GMP, are also cellular second messengers. GTP serves as a precursor of tetrabiopterin, a required cofactor in the synthesis of neurotransmitters, and it is required for signal transduction via G-proteins *(33)*. It is the importance of these nucleotide-dependent processes to functioning and dividing cells that has made the thiopurine antimetabolites so successful.

With the exception of ATP, the intracellular nucleotide pool is small, perhaps 1% or less of the amounts required to synthesize DNA *(34)*. Nucleotide synthesis continues throughout nucleic acid synthesis, and in some cases it may limit the rates of DNA replication and transcription. Rapidly growing erythropoietic cells are geared toward DNA replication and RNA synthesis. These tissues show elevated levels of those enzymes involved in purine and pyrimidine synthesis and a corresponding decrease in the activities of enzymes that degrade nucleotides *(33)*. In the proliferating cancer cell, the activities of the enzymes ribonucleotide reductase, thymidylate synthetase, and inosine monophosphate (IMP) dehydrogenase (Fig. 1) increase as a function of tumor cell growth *(34)*.

2.2.1. Antileukemic Effects

The endogenous nucleotides IMP and GMP regulate the overproduction of purines by feedback inhibition of *de novo* purine synthesis *(33)*. Likewise, the thio analogs (thioinosine monophosphate and thioguanosine monophosphate, respectively) have the same properties *(35)* and could theoretically enhance their own cytotoxicity by reducing endogenous purine production. Thioinosine monophosphate (mercaptopurine nucleotide) can be *S*-methylated by TPMT to form methylmercaptopurine nucleotide, a potent inhibitor of the phosphoribosylpyrophosphate amidotransferase step in purine *de novo* synthesis. Methylmercaptopurine nucleotides are three times as potent as the TGNs at inhibiting this step and over 10 times more potent than methyl-TGNs or mercaptopurine nucleotide *(35)*.

A second control step occurs in the nucleotide interconversion pathway. IMP dehydrogenase is subject to feedback inhibition by the end-point metabolite GMP *(33)*. The 6-thio equivalent (one of the TGNs) could potentiate this decrease in cellular GMP concentrations. IMP dehydrogenase inhibition and decreased intracellular GTP concentrations result in the downregulation of *RAS* and *MYC* oncogenes and the induction of apoptosis in K562 cells *(36)*. The induction of apoptosis linked to perturbations in GTP metabolism may contribute to the thiopurine antileukemic effect. The final act of the thiopurine antimetabolites is incorporation into DNA as a fraudulent base, thioguanine *(31,32)*. Thioguanine residues have been quantitated in DNA derived from leukocytes taken from children undergoing mercaptopurine chemotherapy *(37)*. It has been reported that it is necessary to replace only 0.4% of guanine by thioguanine for antineoplastic activity *(31)*.

2.2.2. Hepatic Toxicity

Thiopurine drugs are potential inducers of liver damage, as reflected in the abnormal liver function tests that are frequently reported in children undergoing maintenance chemotherapy. The sequence of metabolic events that lead to the induction of liver damage are poorly understood. Mercaptopurine is metabolically activated by hepatic cytochrome P450 to purine-6-sulfenic acid, which binds to liver proteins by a mixed disulfide linkage *(38,39)*. Covalent binding of this mercaptopurine metabolite to microsomal protein is irreversible, and when incubated with rat liver microsomal protein, up to 100% of the drug can be protein bound *(38)*. Methylated mercaptopurines have also been implicated in the hepatotoxic effects of thiopurines *(40)*, and children with higher TPMT activities develop more hepatotoxicity *(41)*. In humans, methylmercaptopurines undergo desulfuration *(42)*. The metabolic steps involved in this process have not been fully documented, but methyl-thiol oxidation, with the potential for protein binding, must be considered.

2.3. Drug Interactions

Inhibition of mercaptopurine oxidation or *S*-methylation by concurrent drug therapy will potentiate thiopurine cytotoxicity. Allopurinol is a well-documented inhibitor of xanthine oxidase-catalyzed mercaptopurine oxidation *(43)*. With the mercaptopurine prodrug azathioprine, it is recommended that, during concurrent allopurinol therapy, the azathioprine dose be reduced by two-thirds. This decreases, but does not abolish, the risk of myelotoxicity *(44)*. In the treatment of ALL, allopurinol and mercaptopurine are not used concurrently, but allopurinol and thioguanine are used together in some protocols for acute myeloid leukemia, a possible combination because thioguanine is not a direct substrate for xanthine oxidase *(43)*.

A number of compounds that could be coadministered with thiopurine drugs can potentially influence TPMT activity. However, the drugs listed below, with perhaps the exception of aspirin, are unlikely to be coadministered with thiopurine antileukemic therapy. After a therapeutic dose of aspirin, the plasma concentrations of salicylic acid are within the range for TPMT inhibition *(45)*. Sulfasalazine and its metabolite 5-aminosalicylic acid inhibit TPMT. In a large long-term clinical study of inflammatory bowel disease, mercaptopurine was withdrawn in 10% of patients because of the occurrence of adverse reactions. Over half the patients in that study were treated simultaneously with mercaptopurine and sulfasalazine *(46)*. A possible drug-drug interaction was reported in a patient with refractory Crohn's disease who developed bone marrow suppression while receiving daily mercaptopurine and olsalazine (two molecules of 5-aminosalicylic acid) *(47)*. Concurrent furosemide therapy could influence the *S*-methylation of thiopurines, as furosemide inhibits TPMT with a median inhibitory concentration (IC_{50}) of 170 μM *(48)*, a concentration within the therapeutic range for furosemide. In addition, TPMT could interfere with disulfiram treatment in alcoholism *(49)*. TPMT *S*-methylates the diethyldithiocarbamate metabolite involved in disulfiram activation. Thiopurine drugs themselves have been reported to impair the anticoagulant effect of vitamin K antagonists; the mercaptopurine prodrug azathioprine induces warfarin resistance *(50,51)*.

2.4. Drug Resistance

Thiopurine action may be modulated by factors that influence absorption, active metabolite formation/degradation, and incorporation into DNA. For example, endogenous purine concentrations can influence toxicity. Hypoxanthine at physiologic concentrations protects HL-60, a human acute promyelocytic cell line, from the cytotoxic effects of both mercaptopurine and thioguanine. Increasing the hypoxanthine concentrations threefold required a similar increase in the thiopurine concentration to kill 50% of cells *(52)*. Thiopurine resistance due to absolute HPRT deficiency is rare in human leukemia cells *(53)*, although a 100-fold variation in HPRT activity has been reported in lymphoblasts taken from children with ALL *(54)*.

High cytoplasmic 5'nucleotidase and alkaline phosphatase activities have been associated with thiopurine resistance in some patients *(55)*. Cytoplasmic 5'nucleotidase is responsible for intracellular nucleotide turnover, and together with the nonspecific acid and alkaline phosphatases, could potentially influence intracellular thionucleotide concentrations by dephosphorylation to form nucleosides *(56)*. Thionucleosides can be further degraded by purine nucleoside phosphorylase *(33)* to the parent drug, which could be excreted from or recycled within the cell.

During chemotherapy, one reason for mercaptopurine "resistance" is very high TPMT activities, and extensive methylation results in suboptimal cytotoxic TGN formation *(57)*. A second cause of low TGN concentrations is simply that some children do not take all their medication *(58,59)*. The two problems can be distinguished by the measurement of methylmercaptopurine nucleotide metabolites alongside TGNs *(58)*. Reports based on therapeutic drug monitoring suggest that 10% of children fail to take their mercaptopurine reliably, and this is in agreement with earlier questionnaire-based studies *(58)*.

3. THIOPURINE VARIABLES AND TREATMENT OUTCOME

3.1. Xanthine Oxidase

The influence of xanthine oxidase activity on the outcome of mercaptopurine therapy has yet to be clearly defined, but the fact that allopurinol-induced xanthine oxidase inhibition

potentiates thiopurine cytotoxicity *(43)* indicates a possible role for variable xanthine oxidase activity in treatment outcome. Methotrexate inhibits xanthine oxidase *(60)*, and coadministration of methotrexate with mercaptopurine in children with ALL produces a 31% increase in mercaptopurine plasma concentrations. Because of the variability in mercaptopurine pharmacokinetics, this interaction is thought not to be clinically significant at standard dosages of oral methotrexate *(61)*.

Gender differences have been reported in xanthine oxidase activity, and this oxidative enzyme must not be overlooked as a possible factor influencing the gender difference reported in treatment outcome *(62)*. However, studies using indirect and direct measures of xanthine oxidase activity have produced contrary results. Indirect indices of in vivo xanthine oxidase activity, based on ratios of caffeine metabolites measured in urine, indicate that men have significantly lower enzyme activities than women *(63)*. When xanthine oxidase activity is measured directly in human liver samples, the interindividual variation is fourfold, with men having a 21% higher activity than women *(17)*. Other enzymes, in addition to xanthine oxidase, can contribute to caffeine hydroxylation *(64)*, and this could account for some of the contradictory reports when caffeine metabolism is used as an index of xanthine oxidase activity.

3.2. TPMT

The first indication that TPMT deficiency is associated with profound myelosuppression came from observations in adults taking azathioprine as an immunosuppressive agent. The accumulation of grossly elevated concentrations of mercaptopurine-derived TGN cytotoxic metabolites was linked to a lack of red blood cell TPMT activity *(65,66)*. These observations have since been confirmed by other groups *(67)*.

3.2.1. TPMT and Treatment Outcome

In children with ALL, red blood cell TPMT activity measured at disease diagnosis has been shown to reflect thiopurine metabolism and toxicity during continuing chemotherapy *(68)* and to correlate with leukemic blast TPMT activity *(69)*. A knowledge of before-therapy enzyme activity may help in clinical management, particularly of those children at the extremes of the TPMT frequency distribution with very low or very high TPMT activities. Approximately 400 children/yr enter UK ALL protocols, which specify 2 or 3 yr of antimetabolite-based continuation chemotherapy, so at any one time two or three children taking thiopurines will be TPMT-deficient. Such children can be managed by the use of attenuated dosage regimens (e.g., 10% protocol dose) *(70–72)*.

TPMT-catalyzed thiopurine *S*-methylation competes with TGN formation. At 75 mg/m^2 mercaptopurine, TPMT activities show a significant negative correlation with red blood cell TGN concentrations. Children who develop very high TPMT activities during chemotherapy form low concentrations of TGNs from standard dosages of mercaptopurine. Children with lower TGN concentrations (below the group median) had significantly higher TPMT activities ($p < 0.001$) and a higher subsequent relapse rate *(57)*. This subgroup of children tolerates uninterrupted therapy with full-dose drug and did not experience cytopenias. They are apparently resistant to the cytotoxic effects of mercaptopurine and may form cytotoxic TGN concentrations more reliably and predictably from thioguanine than from mercaptopurine *(73)*. In vitro studies have shown that the more direct intracellular activation pathway for TG results in a higher potency, coupled with the induction of cytotoxicity after a shorter duration of drug exposure, compared with mercaptopurine *(74)*.

3.2.2. TPMT Variablity

*TPMT*3A*, a double mutant, is the most frequently occurring variant allele (*TPMT*^{L}) in white Caucasians, but each mutation can occur independently (*TPMT*3B* and *TPMT*3C*). The *TPMT*3A* mutation appears to have no effect on transcription or mRNA stability, but the resultant very low protein levels indicate that posttranscriptional events result in the loss of functional TPMT activity. *TPMT*2* and *TPMT*3A* proteins show significantly shorter degradation half-lives when compared with the wild-type enzyme *(75)*.

Investigation of other ethnic groups has indicated allelic heterogeneity in the *TPMT* DNA sequence polymorphism. Thus, although *TPMT*3A* is the most common variant allele for low TPMT activity in white populations, other variant alleles exist that may be more common than *TPMT*3A* in other populations. These ethnic differences may be important in the clinical use of thiopurines. In a Korean population, *TPMT*3C* was the most frequent variant allele, and the *TPMT*3A* allele was absent *(27)*. In African-Americans, the mutant allele frequency was the same as recorded in Caucasians, but *TPMT*3C* was the most prevalent mutant allele *(76)*. Similarly, the frequency of *TPMT*3C* was 14.8% in a Ghanaian population, but *TPMT*2*, *-3A*, and *-3B* were not detected *(77)*. Variant alleles were only detected in 2.0% of Southwest Asians (*TPMT*3C*) and 4.7% of Chinese (*TPMT*3C*), compared with 10% of Caucasians (*TPMT*2*, *-*3A*, and *-*3C*), indicating that *TPMT*3C* is the oldest mutation and *TPMT*2* the most recent *(78)*.

Interethnic variability in TPMT phenotype has been documented for a number of ethnic groups *(79–81)*. A Norwegian Saami population had higher, and a North American black population lower, TPMT activities than comparative white populations *(79,81)*. However, the TPMT activity was similarly polymorphic in all three populations. This was not so in a Korean population of healthy children in whom TPMT activity was normally distributed *(80)*.

Long-term and circadian viability in human red blood cell TPMT is low *(82)*. Population studies have shown that the use of diuretics is associated with an increased range of red blood cell TPMT activity *(83)*, but in vitro furosemide was shown to inhibit the enzyme with an IC_{50} of 170 μ*M (48)*, a concentration within the therapeutic range for furosemide. This is not dissimilar to the situation in uremia, which is associated with an increased range of red blood cell TPMT activities, although uremic plasma has been shown to be an inhibitor of TPMT activity *(84)*. Inhibitor stabilization of TPMT against degradation could play a part in this observed increase. Mercaptopurine chemotherapy is associated with an increase in the range of red blood cell TPMT activities *(57)*; by analogy with the observations in uremia, substrate protection against in vivo proteolysis could account in part for this observation. The importance of

proteolysis in TPMT catalytic activity is illustrated by the *TPMT3*A* variant allele. The lack of enzyme activity in this variant allele is not caused by a lack of mRNA but by increased proteolysis of the synthesized enzyme *(75)*.

In population studies of the TPMT phenotype, gender differences in red blood cell TPMT activities have been recorded in some studies *(79,83)* but not in others *(24,57,79,80)*. In hepatic tissue, TPMT activities are significantly higher (13.6%, $p < 0.001$) in men *(85)*. The TPMT frequency distribution and range of enzyme activities are similar in adults and children *(57)*. Mercaptopurine chemotherapy significantly increases the range of TPMT activities by comparison with results in healthy control children, but TPMT activities decrease to the healthy control range at the end of therapy *(57)*. During chemotherapy, the intrapatient variability in red cell TPMT activity is relatively low, but the before-therapy red blood cell enzyme activity was not predictive of the increase in TPMT activity observed during chemotherapy, although red cell TPMT activity before therapy was significantly correlated with the TPMT activity in leukemic blasts at disease diagnosis *(68)*.

A population range for TPMT activity was established in children with ALL at diagnosis as part of the U.K. MRC ALL97 trial. The range of red blood cell TPMT activities measured in 512 leukemic children, prior to treatment, was significantly lower than in a comparative group of healthy children. Circulating red blood cells do not constitute a homogeneous population. Enzyme activities at disease diagnosis could be measured in an abnormally old population of erythrocytes because the anemia of ALL is due to deficient red cell production. Age fractionation of red blood cells has shown that TPMT activities are significantly higher in young compared with older red cells *(86)*. The lower range of TPMT activities recorded at disease diagnosis could therefore simply be caused by an excess of older erythrocytes.

3.2.3. TPMT Phenotype Versus Genotype

TPMT genotyping can, in theory, predict serious myelotoxicity in the 1 in 300 children with TPMT deficiency and can identify those children with an intermediate TPMT activity who may experience a greater frequency of cytopenia-induced thiopurine dose reductions. Genotyping cannot as yet identify those children with very high TPMT activity who may require aggressive upward drug titration before cytopenias are observed, nor can it predict liver or other possible thiopurine-related problems.

In practice, current genotyping techniques can detect 78% *(27)* or more *(87)* of the mutations leading to TPMT deficiency in a Caucasian population. The analysis of variant alleles in a U.K. study of 147 children with ALL identified variant alleles in 11.6% of the children, the allele frequency of the adult British population *(88)*. Apart from one TPMT-deficient child who experienced severe hematologic toxicity, the TPMT genotype did not correlate with, or identify, mercaptopurine hematologic toxicity. Over half the children studied (54%) were treated according to the U.K. protocol UKALL XI, one aim of which is controlled myelosuppression during maintenance in all children. This protocol titrated the mercaptopurine dose to toxicity, and if the child did not respond to 75 mg/m^2 of mercaptopurine, the dose was escalated until cytopenias occurred. The absolute dose prescribed was not investigated with respect to TPMT genotype. It could be that upward titration of mercaptopurine from the standard dose in nonresponders circumvented the TPMT polymorphism.

In long-term treatment of systemic lupus erythematosus with azathioprine, genotyping failed to predict the majority of thiopurine-induced neutropenias *(89)*. In contrast, in rheumatic disease, the TPMT genotype predicted therapy-limiting toxicity induced by azathioprine: five of the six patients with variant alleles experienced leukopenia within 1 mo of starting thiopurines *(90)*.

3.2.4. TPMT Secondary Effects

The possible linkage of secondary effects in survivors of childhood ALL with thiopurine metabolism was recently reported in two separate studies. First, a high incidence of secondary brain tumors (6 of 52 children; 8-yr cumulative risk, 13%) was reported in children surviving ALL who had been treated on an unusually intensive protocol (St. Jude Total XII), which included radiotherapy and concurrent antimetabolite therapy *(91)*. Three of the six children who developed brain tumours had variant (*TPMT*^{L}) TPMT alleles, this subgroup were at the greatest risk (8-yr cumulative incidence, 43%). Four of the six had TGN concentrations above the 70th percentile of the study group. All the children received weekly intramuscular methotrexate (40 mg/m^2) in addition to daily mercaptopurine (75 mg/m^2). Radiotherapy increases the penetration of methotrexate onto the CNS, and methotrexate can also potentiate the effects of mercaptopurine *(91)*. In addition to systemic methotrexate, the Total XII study used a mercaptopurine dose 50% higher than had been used previously with radiotherapy.

This report prompted a review of the rate of secondary malignant brain tumors by other children's centers *(92,93)*. In the United Kingdom, 10 children were diagnosed with malignant brain tumors out of a cohort of 3961 (10-yr cumulative risk, 0.5%) *(92)*. An analysis by the BFM study group indicated that 12 of 1409 children (15-yr cumulative risk, 1.3%) developed CNS tumors *(93)*. In both the U.K. and BFM studies, the mercaptopurine dosage during radiotherapy was lower than the standard continuing chemotherapy dosage, and systemic methotrexate was not given during cranial irradiation. To assess whether TPMT activity was associated with the development of secondary CNS tumors during cranial irradiation in lower dosage mercaptopurine protocols, the frequency of the variant *TPMT2* and *-3* alleles was analyzed in 11 of the 12 children with secondary CNS tumors. Variant TPMT alleles did not determine the risk of secondary CNS tumours *(93)*.

A second report of the association of secondary effects with thiopurine metabolism linked low TPMT activity and high methylmercaptopurine metabolite concentrations with the highest risk of developing secondary myelodysplastic syndrome or acute myeloid leukemia (sMDS/AML) after antileukemic therapy *(94)*. The total dose of cyclophosphamide given failed to reach significance in a Cox model, but the regression analysis implicated both "ends" of the TPMT frequency distribution histogram in the carcinogenic effect of mercaptopurine on bone marrow stem cells. Three of 55 children with a heterozygous

TPMT phenotype and 2 of 384 children with a homozygous wild-type phenotype developed sMDS/AML. The children with a heterozygous phenotype had a 5-yr risk of sMDS/AML of 9%. DNA damage was hypothesized to occur at low TPMT activities due to high concentrations of TGNs, yielding a higher concentration of thioguanine–thymine mismatches in DNA, whereas high TPMT activities produced elevated methylmercaptopurines, which inhibited *de novo* purine synthesis and allowed any available TGNs to be incorporated into DNA *(94)*. TGNs were postulated to interfere with DNA repair mechanisms after DNA damage induced by other drugs. Cyclophosphamide was taken by all children, and two of the five children who developed secondary effects received anthracyclines. Mercaptopurine may have a leukemogenic effect when administered with other cytotoxic agents, and the prevailing immunosuppression may be an additional causative factor *(95)*. There are many long-term studies showing that mercaptopurine alone is not leukemogenic. Indeed, the risk of malignancy following azathioprine or mercaptopurine monotherapy for inflammatory bowel disease is not increased relative to the general population *(96)*.

3.3. Clinical Importance of Thiopurine Metabolites

Despite the widespread use of mercaptopurine for over three decades, many questions regarding the formation and accumulation of its metabolites in various tissues remain unresolved *(97)*. It would be impossible to measure the concentration of thiopurine metabolites in the few remaining malignant cells during maintenance therapy of a child in remission, although this is probably what is important.

Drug metabolism as reflected by mercaptopurine plasma concentrations could provide clinically useful information. Mercaptopurine plasma area under the curve (AUC) can be related to relapse-free survival *(98)* and mercaptopurine peak plasma concentrations to leukopenia *(99)*, but there are pronounced variations between mercaptopurine plasma kinetics when the same patient is studied on repeated occasions *(99)* and there is no correlation between the oral dose of drug and plasma mercaptopurine AUC values *(100)*. Because of this variability, many therapeutic drug monitoring studies have focused on intracellular thiopurine metabolites. Considerable experience has accumulated in the study of circulating red blood cells, as they are plentiful and easy to obtain. To what extent they provide a suitable surrogate is still not entirely clear, but assay of their drug-derived metabolites can provide useful clinical information.

3.3.1. Red Blood Cell TGNs in Children on Mercaptopurine

In most individuals (>99%), cytotoxic TGNs accumulate slowly within the red blood cells on a time scale of days and weeks rather than hours *(101)*. Conversely, in the TPMT-deficient individual, TGNs accumulate rapidly and continue to accumulate up to 7 d following a single oral dose of mercaptopurine *(72)*. During this period of continued TGN accumulation, plasma levels of mercaptopurine are below the limits of detection. These observations raise questions as to the source of the TGN metabolites. They could be formed entirely within red blood cells during a course of therapy and/or come from mercaptopurine metabolites originating elsewhere.

The study of red cell TGN concentration during maintenance as a predictor of outcome was first attempted a decade ago in a small cohort of children from the English East Midlands. When prescribed the same mercaptopurine dose based on body surface area, children varied widely in the concentration of red cell drug metabolites. Red cell TGN accumulation, studied from the first mercaptopurine dose, was lower in those children taking uninterrupted 75 mg/m^2 mercaptopurine than in children undergoing frequent dose reductions *(101)*. To investigate whether this variation mattered in terms of disease control, red cell TGNs were measured in 172 first remission children. There was a 12-fold range in TGNs (113–1340 pmol/8×10^8 red cells; median, 284) at 75 mg/m^2 mercaptopurine, and children who failed to form higher amounts of TGNs were at an increased risk of disease relapse *(101,102)*. Multivariate analysis confirmed that the effect of the TGNs on disease control was independent of other prognostic factors.

It is important to understand, however, that the group studied was treated on less intensive protocols than those used in later years, and physicians were less inclined to titrate the dose of mercaptopurine to produce myelotoxicity. Since then there has been a trend to more aggressive prescribing in the United Kingdom, and median red cell TGN levels measured in children on mercaptopurine have steadily risen over the intervening years. This might explain why subsequently treated children have not shown the same phenomenon, and why the findings have not been confirmed in more recent studies in the United States *(14,103)* although a Nordic study did find that children with values below the median concentration of erythrocyte methotrexate polyglutamates multiplied by that for TGNs had a significantly poorer event-free survival *(104)*.

In Crohn's disease patients on long-term mercaptopurine therapy, red cell TGN levels show a significant correlation with the amount of thioguanine incorporated into leukocyte DNA *(105)*. In renal transplant recipients treated with azathioprine, those who accumulated TGN at >200 pmol/8×10^8 red cells developed significantly lower neutrophil counts than those who did not *(106)*. In this patient group, neutrophil methylmercaptopurine metabolites were undetectable, and neutrophil TGN concentrations were 30-fold higher than red cell TGNs *(107)*.

3.3.2. Red Cell TGNs in Children on Thioguanine

There is a difference in the pattern of erythrocyte metabolite accumulation between mercaptopurine and thioguanine, with the latter generating much higher concentrations of TGNs at isotoxic doses *(108,109)*. Mercaptopurine undergoes extensive oxidative metabolism, and both mercaptopurine and its intermediate metabolites are readily methylated by TPMT. Thioguanine undergoes less extensive intermediary metabolism (*see* Section 2.1.). The sevenfold or more increase in red cell TGN concentrations measured in children on thioguanine reflects the ability of the red cells to metabolize thioguanine to TGNs directly, whereas red cells form TGNs from mercaptopurine with difficulty, and there is strong evidence that mercaptopurine-derived TGNs are obtained, at least in part, from other tissues *(97)*.

The clinical importance of red cell TGNs in children taking thioguanine is unclear and may be minimal or nonexistent apart

from monitoring patient compliance. There is no clear correlation with myelosuppression, as has been observed in children taking mercaptopurine, but a TPMT-deficient child treated on the MRC ALL97 protocol and randomized to thioguanine accumulated TGNs at 13,904 pmol/8 × 10^8 red blood cells after 2 wk of treatment with 40 mg/m^2. A 2-mo period of prolonged, profound myelosuppression followed. In the UK ALL97 trial, TGN concentrations observed in children with TPMT activity taking 40 mg/m^2 thioguanine ranged from 0 to 3808 pmol/8 × 10^8 red cells (median, 1844) *(110)*.

Preliminary work comparing TGN concentrations in peripheral blood leukocytes vs red cells, despite the technical difficulties involved, suggests that the huge difference in red cell TGN concentrations observed in children taking thioguanine vs mercaptopurine is not seen in leukocytes. For children on either mercaptopurine or thioguanine, leukocytes contain more TGNs, cell for cell, than do erythrocytes, but the difference is much greater for children on mercaptopurine—10-fold or more—compared with only 2–5-fold for children on thioguanine *(111)*.

3.3.3. Methylated Metabolites

A number of methylated thiopurine metabolites have been measured in vivo including the parent thiopurine, its nucleotide and the respective 8-hydroxy nucleotides *(18,58,112)*. Methylmercaptopurine nucleotides, like elevated TGNs, are potent inhibitors of *de novo* purine synthesis. Methyl-TGNs, like mercaptopurine nucleotides, are less effective in this inhibition *(35)*. Children taking mercaptopurine have much higher methylmercaptopurine nucleotides in their red cells compared with the TGNs. Conversely, children taking thioguanine have much higher TGNs than methyl-TGNs. Methylmercaptopurines are lacking in children taking thioguanine, whereas methyl-TGNs are not detected until the TGN concentrations exceed 500 pmol/8 × 10^8 red cells *(113)*. Grossly elevated methylmercaptopurine levels are observed in children with very high TPMT activities during mercaptopurine therapy *(58,114)*. These children do not develop cytotoxicity at standard mercaptopurine dosages *(57)*, but rather require dose escalation before neutropenia is observed *(115)*.

The roles of the methylthiopurines in vivo are uncertain; methylmercaptopurine nucleotides can inhibit the synthesis of purines *de novo* and therefore potentiate the effects of any thiopurine analogs. Methylmercaptopurine nucleotides have also been implicated in the hepatotoxic effects of thiopurines *(40,41)*. The ability of TPMT to deplete intracellular concentrations of the universal methyl donor *S*-adenosyl-L-methionine may influence cellular mechanisms at many different levels *(116)*.

3.3.4. Compliance

The study of thiopurine metabolites in red cells has drawn attention to a previously overlooked problem—that some children do not fully comply with their prescribed medication. The tacit assumption that the life-threatening nature of ALL ensures full compliance is clearly wrong *(59,117)*. TGNs accumulate in red cells over days to reach or approach steady state in most patients taking a constant dose *(101)*. The final TGN concentration is under the influence of several variables, the most important of which are TPMT activity, dose prescribed, and dose taken. Variation within a wide range is therefore to be expected, but if a standard protocol dose is prescribed over a period of weeks or months, it is difficult to explain why some children have barely detectable or wildly fluctuating concentrations of red cell TGNs on any basis other than poor compliance *(118,119)*.

The problem may arise in 10–20% of children in the United Kingdom *(58)* and may be a particular problem with adolescents *(119)*. In North America, electronic mediation monitoring has indicated that 17% of children take <80% of their prescribed mercaptopurine *(120)*. What effect this has on long-term outcome is not yet clear, but it is hard to imagine that it is irrelevant. The high relapse rate in developing countries is probably amplified by failure to take maintenance therapy following discharge from the hospital *(121)*, and outcome has always been inferior where poverty, malnutrition, and poor parental education are prevalent—all risk factors for poor compliance *(122)*. Monitoring drug metabolite concentrations does provide an opportunity to detect potential poor compliers at an early stage and to intervene; in the end, this may prove to be their most important clinical application.

3.3.5. Which Is the Better Thiopurine?

Custom and practice have dictated that mercaptopurine be used in ALL maintenance, but there is no sound pharmacologic reason for this *(73)*. In vitro data show that ALL cells are more sensitive to thioguanine than mercaptopurine *(123,124)*, and the different way the two drugs are metabolized makes it possible that higher concentrations of cytotoxic nucleotides will be available from thioguanine. The two drugs produce a roughly isotoxic effect in terms of myelosuppression only when the dose of mercaptopurine is nearly twice that of thioguanine *(109)*. Thus so a direct comparison in randomized trials is of interest.

Three such trials are either recently completed or continuing: COALL 05-92 in Germany *(125)*, Children's Cancer Group (CCG) 1952 in North America *(126)*, and ALL97 in the United Kingdom. The COALL trial closed in July 1997 after accruing 480 randomized children over 5 yr. Interim analysis after a minimum follow-up time of 2 yr showed no benefit for those receiving thioguanine. One contributory factor might have been the fact that those receiving thioguanine had seven times more frequent treatment interruptions due to isolated thrombocytopenia. The CCG study closed to new entrants in late 1999, and the U.K. study closed in mid 2002. The U.K. trial had sufficient accrual (circa 2000 patients) to answer the question with a 65% chance of detecting a 5% difference in event-free survival, the power to detect a 10% difference will be 99%.

A toxicity encountered in both the CCG study and the U.K. trial has been that of mild reversible venoocclusive disease of the liver in 3–6% of children taking thioguanine, a complication not observed in the COALL children. The syndrome is characterized by abdominal pain, hepatomegaly, ascites, thrombocytopenia, and relatively modestly raised bilirubin and deranged liver enzymes *(127)*. It regresses on withdrawal of thioguanine, and subsequent mercaptopurine can be tolerated without problems. It may be related to high doses of thiogua-

nine for prolonged periods *(126)*. It has been reported in renal transplant recipients taking azathioprine after renal transplantation *(128)*. Thus the indications are that thioguanine could have a worse toxicity profile, although it may prove superior in terms of leukemia control in some children. This latter point will not become clear until the data from all three of the aforementioned studies have become mature.

4. THIOPURINE DOSE

4.1. Dosage Adjustments

In thiopurine therapy, efficacy and toxicity are interrelated; myelosuppression is required for a successful anticancer effect *(57)*. The aim of treatment with thiopurine drugs in the UK ALL protocols is controlled myelosuppression, and during continuation chemotherapy, myelosuppression is used as a surrogate measure of response to thiopurine therapy. The UK ALLVIII trial showed that strict mercaptopurine dosage criteria, whereby mercaptopurine was titrated to myelosuppression, albeit to a protocol maximum of 75 mg/m^2, produced an otherwise unexplained 20% improvement in disease-free survival over previous "gentle" protocols *(129)*. This implies that some children require more mercaptopurine to induce cytotoxicity, and the results from the UK ALLVIII trial support observations with respect to inherited TPMT activity and treatment outcome *(57)*. For those children who do not respond to the protocol's standard mercaptopurine dose (75 mg/m^2, 100% dosage), dose escalation in 25% increments every 4 wk increases TGN concentrations and induces neutropenia *(115)*. However, for the limited number of children whose thiopurine dose was escalated to 150%, measurement of mercaptopurine metabolites indicated compliance problems *(58,115)*.

In the TPMT-deficient child, 100% daily doses of thiopurines produce profound, life-threatening myelosuppression. A pharmacokinetic study of a TPMT-deficient child taking 75 mg/m^2 (100%) mercaptopurine once a week showed continued accumulation of TGNs in the 7 d following the single oral dose. After four doses at weekly intervals, red cell TGN concentrations were double the upper end of the TGN range observed in children with TPMT receiving daily mercaptopurine *(72)*. The TGNs had a half-life of 12 d and a terminal half-life of 25 d. At 10% daily dosage, this child experienced unacceptable gastrointestinal symptoms; adjustment to 10% on alternate days eliminated these toxic effects, but TGN levels remained elevated *(72)*. These concentrations are just above the upper limit of the range of TGN metabolites measured in children with TPMT activity, and they are similar to the range of TGN concentrations measured in children with ALL receiving thioguanine as an alternative thiopurine to mercaptopurine *(73)*.

In vitro studies of hepatic mercaptopurine metabolism in human liver incubates *(97)* indicate that increasing mercaptopurine concentrations increase the rate of drug *S*-methylation and oxidation. This is in keeping with the K_M values for the metabolism of mercaptopurine by xanthine oxidase and aldehyde oxidase (approx 100 μ*M*) and the K_M value of hepatic TPMT (580 μ*M*) *(25,97)*. This could have implications for oral dosage schedules. A single high dose of mercaptopurine (1500 mg) produced no adverse effects in an individual who had been taking 75 mg mercaptopurine daily for 2.5 yr *(130)*. No exacerbation of the existing hepatitis and no adverse reaction to the overdose was noted. Similarly, after a massive dose of azathioprine (7500 mg), the toxicity was low *(131)*. These observations are compatible with increased catabolism of the drug at high concentrations. Chronically administered low doses of mercaptopurine are frequently toxic *(102)*. Thionucleotide metabolite formation is very efficient at low drug dosages; the K_M of red cell HPRT for mercaptopurine is 19 μ*M* *(132)*. Dosage and dosage interval may determine the amount and persistence of active thionucleotide metabolites in target cells. It could be speculated that, for those children taking mercaptopurine during continuing chemotherapy who do not respond to the standard dosages (75 mg/m^2), multiple daily split dosages could have benefits over a single elevated dose.

4.2. Modifications for Hepatotoxicity

Abnormal liver function tests are frequently reported in children undergoing thiopurine maintenance chemotherapy. Although three out of four children with ALL can now look forward to long-term disease-free survival, recurrent disease remains a major risk for 25% of children, and the withdrawal of thiopurine therapy in response to hepatotoxicity could do more harm than good. A degree of hepatotoxicity has been postulated to reflect the magnitude of systemic drug exposure and could be a favorable prognostic factor *(133)*. A population-based study of ALL confirmed the high prevalence of hepatotoxicity in children receiving antimetabolite maintenance therapy, with 60% of the 115 children studied having serum aminotransferase levels indicative of persistent hepatotoxicity. Cox's proportional hazard regression analysis indicated in this group of patients that hepatotoxicity was associated with a reduced relapse risk. This study concluded that dose reductions are not justified in the face of hepatotoxicity unless liver damage is confirmed by biopsy *(133)*. The development of venoocclusive disease during thioguanine therapy (*see* Section 3.3.5.) is treated by stopping thioguanine and, once biochemical and hematologic recovery has occurred, switching to mercaptopurine.

Intermittent jaundice during a course of chemotherapy among Japanese, Koreans, and Chinese may be caused by other metabolic factors. A mutation common in these Asian populations results in suboptimal hepatic bilirubin UDP glucouronosyltransferase activity, a deficiency that can be a precipitating factor in the development of liver dysfunction in children with ALL *(134)*. The intermittent hyperbilirubinemia is mild, with no structural liver damage.

4.3. Oral Versus Intravenous Dosage

Forty years after the effectiveness of low-dose oral mercaptopurine was demonstrated in ALL, intravenous mercaptopurine was developed as an investigational drug *(135)*. Studies on low-dose oral mercaptopurine suggested that there was considerable intrapatient variability in plasma drug and intracellular mercaptopurine metabolite concentrations. Furthermore, inadequate exposure to mercaptopurine and/or its metabolites was associated with risk of disease relapse *(98,101)*. In vitro studies had shown that optimal mercaptopurine cytotoxicity was achieved by prolonged exposure to higher concentrations of

mercaptopurine than had been observed during oral administration *(135)*. These observations provided some of the impetus behind the initial development of high-dose intravenous mercaptopurine strategies. The reasoning was that a higher dosage would result in higher, less variable systemic exposure to mercaptopurine. In addition, it may be possible to increase CNS exposure to mercaptopurine during intravenous dosage *(135)*. The major use of intravenous mercaptopurine, together with intravenous methotrexate, is in postremission intensification protocols *(136,137)*. One potential advantage of high-dose mercaptopurine is that its use avoids therapies associated with a greater therapeutic hazard. CNS mercaptopurine concentrations are elevated 21-fold compared with those attained during oral maintenance therapy, although it has yet to be demonstrated whether this reduces the incidence of CNS relapse *(138)*, and the unique value, if any, of high-dose intravenous thiopurines is not yet established in ALL.

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ANTILEUKEMIC DRUGS

III

D

EPIPODOPHYLLOTOXINS

31 Etoposide in Acute Leukemias

Therapeutic and Pharmacokinetic Considerations

MARY V. RELLING

CONTENTS

1. INTRODUCTION

The role of etoposide in treating acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) is controversial *(1–4)*. From the earliest investigations, it has been clear that etoposide possesses antitumor activity against a wide variety of solid tumors, lymphomas, and leukemias *(5)*. However, randomized comparisons of etoposide vs other agents in acute leukemias are relatively rare. Because of the undesired leukemogenic potential of etoposide, it is particularly important to identify which patients are most likely to benefit from its use.

2. IDENTIFICATION OF LIKELY PATIENTS

2.1. Acute Lymphoblastic Leukemia

The single-agent activity of etoposide was demonstrated in phase I–II trials in childhood ALL patients, with several complete responses reported for this agent *(6)*. The published response rates to etoposide as a single agent have generally been better for AML than for ALL, although this discrepancy has been attributed to heavier pretreatment of ALL patients enrolled on phase I–II trials *(4)*.

Although the improvement in cure rates for childhood ALL to the 70–80% range coincided temporally with the incorporation of etoposide into multiagent regimens in some therapies *(7–10)*, this agent was never the only change in those regimens, making it impossible to attribute the improvement in cure rate to etoposide alone. Moreover, some childhood ALL protocols have produced long-term event-free survival (EFS) rates in the 75–80% range without the addition of etoposide *(11,12)*, relying instead on more intensive use of anthracyclines or asparaginase, or cranial irradiation for high-risk groups. Because the role for etoposide in childhood ALL is not clear, and overall survival is good, most modern frontline treatment protocols include an epipodophyllotoxin such as etoposide only for standard or higher-risk ALL patients *(13)*. Epipodophyllotoxins have been prominent components of effective multiagent salvage chemotherapy for relapsed ALL *(14)* and thus may play an important role in the relapse setting.

The role of epipodophyllotoxin therapy in adult ALL is not clear *(15–18)*, although results have generally been disappointing.

2.2. Acute Myeloblastic Leukemia

Because of the poor overall EFS rates in AML, the primary focus has been on whether etoposide is efficacious, and leukemogenicity looms less daunting a concern than it is for the treatment of ALL. A summary of single-agent activity indicates an overall 17% complete remission (CR) rate in 233 pediatric and adult AML patients, most of whom were heavily pretreated *(3)*. When etoposide was combined with cytarabine plus an anthracycline in newly diagnosed AML, CR rates ranged from 48 to 86% *(3,4)*. A randomized trial of anthracycline and cytarabine with or without etoposide indicated no difference in CR or overall survival, but etoposide was

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associated with a significantly longer remission duration *(1)*. In the Medical Research Council (MRC) AML10 randomized trial, adults with newly diagnosed AML received daunorubicin and cytarabine plus either etoposide (100 mg/m^2 over 1 h, d 1–5) or oral thioguanine for 10 d *(19)*. There were no differences in any assessment of efficacy between the two arms and no suggestion of improved activity of etoposide in monocytic subtypes of AML, which has been suggested by some *(4)*. Taking these data together, it appears that addition of etoposide to standard anthracycline/cytarabine adds to the antileukemic effect in newly diagnosed AML, but it is not clear that it is more or less effective than other "third" agents. In relapsed AML, there were no differences in survival or remission rates in a randomized trial of diaziquone plus mitoxantrone, mitoxantrone plus continuous-infusion etoposide, or diaziquone plus continuous-infusion etoposide *(20)*.

Etoposide has often been incorporated into bone marrow preparative regimens *(21–23)*. In this setting, hematologic rather than gastrointestinal toxicity is preferred, and by administering short infusions of high doses rather than prolonged infusions, mucositis from such high doses has been acceptable. The plasma concentration of etoposide just prior to stem cell infusion has been positively correlated with a delay in engraftment among autologous transplant patients, and the anticonvulsants that are often given during stem cell transplant preparative regimens are associated with increased etoposide clearance *(24)*. Conditioning regimens including etoposide are associated with an increased risk of therapy-related acute myeloid leukemia (t-AML) with 11q23/21q22 abnormalities *(25)*.

In summary, as for many antileukemic agents, the importance of etoposide in the treatment of leukemia relative to other effective agents is not clear.

3. WHAT SCHEDULE OF ETOPOSIDE ADMINISTRATION IS BEST FOR TREATING LEUKEMIA?

3.1. Acute Lymphoblastic Leukemia

The overall EFS at 4 yr in an ALL trial with a high incidence of t-AML (5.9%) was excellent, 79.3% *(26)*. Moreover, EFS was comparable in two arms of the St. Jude Children's Research Hospital (SJCRH) Total XI study (72% vs 70%) *(27)*, one of which was associated with a much higher incidence of t-AML (12.4% vs 1.5%) *(28)*. These findings raise the disheartening possibility that the most efficacious etoposide schedules are the most leukemogenic ones, although there are some in vitro data to indicate that cytotoxicity and sublethal nonhomologous recombination are not necessarily inextricably linked *(29)*.

3.2. Acute Myeloblastic Leukemia

There are few data indicating an optimal schedule for etoposide administration in AML. In the one randomized study that showed a benefit of etoposide *(30)*, the drug was given at 75 mg/m^2/d as a short 1-h infusion daily for 7 d.

4. WHAT SCHEDULE IS BEST FOR AVOIDING T-AML?

The topic of t-AML induced by topoisomerase poisons has been reviewed *(31–37)*. When investigating whether t-AML is "schedule-related" or not, one must consider several aspects: the frequency of administration (every day for several days vs once every 1, 2, or 3 wk); the acute method of administration (e.g., short infusion vs long infusion vs oral administration); and the other therapy that is given prior to, concurrent with, or after etoposide. Of course, there are few randomized trials comparing different etoposide schedules, so inferences regarding schedule are fraught with limitations.

4.1. Frequency of Administration

An underlying principle should be kept in mind: a hematopoietic progenitor that is killed by exposure to etoposide (with or without other antineoplastics) is not a candidate for leukemogenic transformation. In contrast, a hematopoietic progenitor with sublethal DNA rearrangements induced by etoposide (with or without other antineoplastics) is a candidate for transformation. Thus, schedules that are highly and acutely cytotoxic are not necessarily particularly leukemogenic, whereas very modest exposures to etoposide may not provide a potent enough recombinogenic "hit" to be leukemogenic.

The SJCRH Total XI protocol provided a concurrent comparison of two different schedules for administration of epipodophyllotoxins, always combined with cytarabine or cyclophosphamide, in newly diagnosed ALL *(28,38)*. One group of patients was randomized to receive blocks of once-weekly × 6 wk of the epipodophylloxin combination, whereas the other group received the exact same weekly doses of drugs, but the combinations were rotated weekly, so that different combinations were given every week (Fig. 1). Overall EFS was identical in the two arms *(27)*, but there was a higher frequency of secondary AML in the weekly × 6 group and thus a higher ALL-free survival effect in the every-other-week group.

From analysis of the combined data from SJCRH protocols Total XI and X, two schedules of administration were deemed quite leukemogenic, with the weekly × 6 and the twice-weekly × 2 schedules being implicated in t-AML *(28)*. Although these data suggested that "frequent administration" might be more leukemogenic, there are in fact several reports of daily × 5 etoposide schedules that are associated with low rates of t-AML *(39)*, and thus a simple analysis of frequency of dosing is not necessarily informative.

4.2. Acute Method of Administration

The impact of acute dose intensity is difficult to assess: no differences (and very low incidences) of t-AML were reported among patients with testicular cancer who received etoposide at 100 mg/m^2/d × 5, 200 mg/m^2/d × 5, or 400–500 mg/m^2/d × 3 *(40)*, although concurrent chemotherapy with varying schedules of alkylating agents confounds any assessment of acute dose intensity. In a summary of four trials in patients with germ cell tumors, the trial with the highest incidence of t-AML was not the one with the highest cumulative dose but was the one with the lowest amount of etoposide per cycle *(41)*.

Varying results have been reported for daily × 5 schedules. In a review of trials with most etoposide doses at 75–100 mg/m^2/d × 5, often combined with an alkylator, risks of t-AML were relatively low, but most cases of t-AML did not display the *MLL* rearrangements that typify etoposide-induced AML cases *(39)*. Daily × 4 schedules of 200 mg/m^2 given to

children with lymphoma were followed by no cases of t-AML, whereas the same daily dose given twice per week (albeit with asparaginase and thiopurine therapy) resulted in an 18.4% incidence of t-AML *(42)*. However, a relatively high incidence of t-AML of 8% was reported *(41)* with a daily × 5 schedule when etoposide was combined with intensive ifosfamide and followed by granulocyte colony-stimulating factor.

We found that the frequency of recombinogenic events induced by etoposide in human lymphoblastoid cell lines was greater with short exposures to high concentrations (mimicking an iv infusion over 4 h) than it was with longer exposure to lower concentrations (mimicking every 8–12 h oral dosing or a prolonged infusion) *(29)*. We hypothesized that oral administration might be less leukemogenic than short iv infusions. Subsequently, there have been several reports of t-AML among patients who received primarily oral etoposide *(43–46)*. However, most of those cases involved radiation or other antineoplastics, and most have not involved 21-d schedules of oral etoposide (which are relatively cytotoxic) but instead involved intermittent 5–14-d schedules that may be nontoxic enough to allow sublethal rearrangements to persist.

4.3. Concurrent Therapy

For any schedule, it is easy to envision that the concurrent therapy plays a critical role in the effects of etoposide on the hematopoietic progenitors and thus on the risk of t-AML.

Our own data from the Total XI study (Fig. 1) *(28,37,47)*, in addition to data from other groups *(26,42)*, can be interpreted to indicate that periods of chronic antimetabolite (mercaptopurine and methotrexate) preceding epipodophyllotoxin therapy may predispose to t-AML. In a comparison of pharmacologic risk factors for t-AML, low thiopurine methyltransferase (and thus high exposure to thiopurine active metabolites) predisposed to t-AML *(48,49)*. In fact, when etoposide was used to treat Langerhans cell histiocytosis, a higher incidence of t-AML was observed when etoposide was combined with "drugs not considered to have a significant leukemogenic effect," such as methotrexate and mercaptopurine, than when it was combined with alkylators, radiation, or intercalators *(50)*, providing further support for the potential role of antimetabolites in facilitating the leukemogenic properties of etoposide. There are several mechanisms whereby thiopurines could enhance epipodophyllotoxin-induced t-AML *(48)*.

Asparaginase pretreatment potentiates the risk of t-AML *(51,52)*. Although the mechanism for this potentiation is not clear, our pharmacokinetic data suggest that the higher exposure to unbound etoposide or its catechol metabolite due to asparaginase's inhibition of hepatic protein synthesis *(53)* is unlikely to be the mechanism *(48)*. Perhaps asparaginase-induced inhibition in the synthesis or function of some protein(s) involved in protection from etoposide-induced recombinogenesis is contributing.

Alkylators may also predispose to etoposide-induced t-AML. A highly leukemogenic schedule of etoposide plus cisplatin in patients with non-small cell lung cancer involved weekly doses of etoposide at 300 mg/m^2 per dose *(54)*, which could be interpreted to implicate frequent etoposide administration (more than once per 2 wk) or to implicate the combination with the alkylator cisplatin.

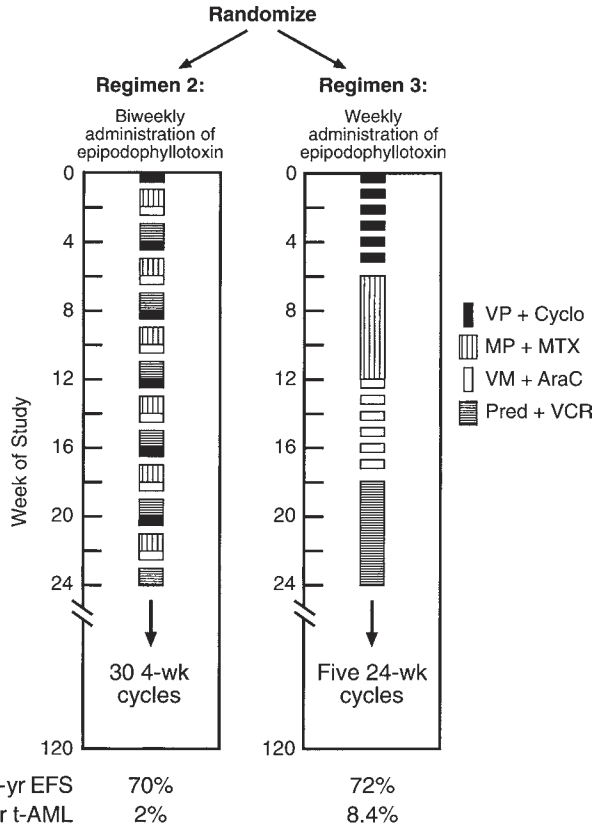

Fig. 1. Schema of continuation therapy in St. Jude Children's Research Hospital Total XI study for higher risk patients, in which identical pairs of antineoplastics were given weekly by rapidly rotating the pairs every week (regimen 2, left side) or in blocks of 6 consecutive wk for each pair (regimen 3, right side). The similarity in overall event-free survival (EFS) contrasts with the vastly different cumulative incidences of therapy-related acute myeloid leukemia (t-AML) for patients randomized to the two arms (bottom). VP, etoposide; Cyclo, cyclophosphamide; MP, 6-mercaptopurine; MTX, methotrexate; VM, teniposide; AraC, cytarabine; Pred, prednisone, VCR, vincristine.

4.4. Cumulative Dose

Interestingly, although the cumulative dose of etoposide has sometimes been associated with risk of t-AML *(38,54)*, there are strong data to indicate that cumulative dose is unlikely to be important, except of course in the sense that each subsequent dose translates into another chance for a leukemogenic event. A prospective monitoring plan for t-AML among cooperative group clinical trials failed to demonstrate any relationship between cumulative epipodophyllotoxin dose and t-AML *(39,55)*. In a summary of four trials in patients with germ cell tumors, the trial with the highest incidence of t-AML was not the one with the highest cumulative dose but was the one with the lowest amount of etoposide per cycle *(41)*. The cumulative incidences of t-AML were 18.4 and 0% on two multiagent lymphoma protocols with cumulative etoposide doses of 5600 vs 10,000 mg/m^2, respectively *(42)*, with the high frequency associated with the lower cumulative dose. (Interestingly, the

former involved intensive mercaptopurine and asparaginase concurrent with etoposide.) Our own data for concurrently randomized patients in the Total XI study indicate no relationship between cumulative dose and risk of t-AML *(28)*. Cases of t-AML with *MLL* rearrangements have been demonstrated after cumulative doses of as little as 400 mg/m^2 in a single course *(56)*, suggesting that the leukemogenic event can be induced with any etoposide exposure.

In summary, schedule, concurrent therapies, method of administration, and host factors *(48,49,57,58)* may all contribute to the risk of etoposide-induced AML, with some factors apparently outweighing others in different clinical trials. One must assume that even minor changes to a previously demonstrated "safe schedule" may tip the balance in favor of leukemogenesis and that the occasional strongly predisposed individual may develop t-AML despite our best efforts to control the schedule of administration.

5. ORAL VERSUS SHORT IV VERSUS PROLONGED IV METHODS OF ADMINISTRATION: SPECIAL CONSIDERATIONS

Etoposide is cytotoxic, and its leukemogenic effects are probably cell-cycle specific. In vitro, cytotoxicity was related to total exposure to etoposide, regardless of the length of exposure *(59)*. However, the shape of the blood concentration vs time curve in vivo appears to be an important determinant of efficacy, as a single daily dose of 500 mg/m^2 iv was less effective in patients with lung cancer than were daily doses of 100 mg/m^2 iv for 5 d *(60)*, even though the total area under the curve (AUC) was identical in the two groups. It also appears that there is some minimum threshold level of systemic exposure that is required for a cytotoxic effect in vivo *(61)*, and this level is higher than concentrations that are cytotoxic in vitro, partly because of higher protein binding in vivo compared with in vitro *(62,63)*. Although it might be tempting to speculate that more prolonged infusions resulting in longer time periods above a threshold should always be more effective, the potential advantage of short exposure to high concentrations in terms of securing higher drug levels in sanctuary sites (central nervous system and testes) *(64)* and in acting in concert with other concurrent chemotherapy cannot be ignored. In addition, there are several examples in adults with solid tumors showing similar activity with prolonged infusions vs divided doses *(65)*.

Short infusions of high-dose etoposide have been relatively well tolerated; in children, such infusions of teniposide must be given with caution owing to the high concentrations of ethanol and Cremophor in the formulation *(66)*.

Etoposide displays dose-dependent absorption, with a lower fraction absorbed at 400 mg/m^2 than at 50–100 mg/m^2 *(67,68)*. Thus, it has been suggested that doses >50 mg/m^2/d be divided into two or three daily doses.

Because of the relatively high frequency of acute reactions to etoposide *(69)*, prolonging the infusion from 1 h to 3–4 h may be attempted to minimize the dose infused and possibly the severity of the reaction, should one occur. However, prolonging an infusion from 1 h to 4 h will reduce the peak concentrations by a factor of 4 (although the time above a moderately high concentration will increase), and so altering the infusion length may affect efficacy and toxicity. Although the induction of mucositis *(20)* rather than hematopoietic toxicity may be dose-limiting with prolonged compared with short infusions, randomized trials to resolve this issue are lacking. With divided (three times daily) oral dosing, gastrointestinal toxicity was dose-limiting in children.

6. CAN ETOPOSIDE BE USED IN PATIENTS WITH RENAL OR LIVER FAILURE?

Etoposide undergoes excretion unchanged in the urine, hepatic metabolism to inactive and active metabolites, and biliary excretion as both unchanged drug and metabolites *(61,70)*. With 72-h iv infusions, the median percentage of clearance accounted for by renal clearance was 13–17% *(71)*, whereas 60% of short iv infusions may be renally cleared *(61,72)*. In general, especially with non-bolus dosing, renal elimination may "compensate" for poor hepatic elimination and vice versa *(73)*. Thus, there are examples of etoposide being given safely to patients with either hepatic *(73,74)* or renal *(75)* failure, but not both, and generally not in the presence of hypoalbuminemia. Moreover, smaller doses tend to be associated with normal clearance, no matter what type of organ dysfunction exists, possibly because other pathways of clearance may "compensate" for the diseased pathway if not saturated by high doses.

The impact of liver failure on etoposide disposition is somewhat difficult to assess. Total (unbound plus bound) etoposide disposition did not differ in patients with and without hyper-bilirubinemia *(73,74,76)*. This is because hepatic failure is frequently associated with hyperbilirubinemia or hypoalbuminemia, both of which result in lower etoposide plasma protein binding *(63)*, but often hepatic failure (as defined by elevated transaminases and/or hyperbilirubinemia) is not necessarily associated with decreased synthetic function or decreased activity of drug-metabolizing enzymes. In the absence of decreased unbound intrinsic clearance of the liver, a higher free fraction of drug is accompanied by higher total drug clearance but results in no net change in exposure to unbound active drug. Some have found liver failure to be associated with higher exposure to free drug and greater toxicity *(77,78)*, whereas others (studying low doses of 50–100 mg) have not *(74)*. Hypoalbuminemia can also be associated with poorer tolerance to etoposide therapy *(79)*.

Doses of 25–250 mg etoposide have been administered to patients with dialysis-dependent renal failure with normal kinetics and toxicity *(75,80,81)*, and etoposide is not cleared by hemodialysis *(80,82)*. However, there are examples of etoposide clearance correlating negatively with serum creatinine *(74,83,84)*, indicating the need for caution while administering etoposide to patients in renal failure, especially with higher doses over relatively short infusion times or with concurrent drugs that would compete for hepatic elimination, or when the patients have coexisting hepatic dysfunction or hypoalbuminemia.

Because both liver and kidney function may be important for etoposide elimination, interaction with drugs that interfere

with either should be anticipated. Epipodophylloxin clearance is increased by CYP3A4 inducers *(24,85)*, and although the drugs have CYP3A-formed active metabolites *(86–89)*, evidence suggests that such increased clearance may be associated with reduced activity *(90)*. Likewise, cisplatin use, which may decrease synthesis of the P450s metabolizing etoposide and decrease glomerular filtration rate, is associated with reduced etoposide clearance *(71,91)*.

7. ARE THERE GENETIC POLYMORPHISMS AFFECTING ETOPOSIDE PHARMACODYNAMICS?

The role of genetic polymorphisms in drug-metabolizing enzymes, drug transporters, and drug targets is becoming more widely appreciated *(92)*. Etoposide systemic clearance displays a unimodal frequency distribution in every population thus far studied, indicating that there are unlikely to be polymorphisms in major genes determining its pharmacokinetics that are subject to completely inactivating mutations. However, polymorphisms in regulatory elements, such as promoter single-nucleotide polymorphisms (SNPs), or SNPs in the coding regions that result in modest amino acid substitutions, could play a role in determining some of the interpatient variability in etoposide disposition.

Etoposide is metabolized by CYP3A4 and CYP3A5 *(86,87,93)*. A polymorphism in the 5' regulatory element for *CYP3A4 (94,95)* has been identified, with the variant present at strikingly different frequencies in American white vs black populations *(58,96)*. Although the functional significance at the catalytic activity level is controversial, the polymorphism has already been linked to the risks of t-AML and other cancers *(58,94)*, and so it may have some importance for etoposide effect. Coding region *CYP3A4* variants have also been reported *(96)*.

Etoposide is a substrate for P-glycoprotein, which has implications not only for drug resistance in tumor cells but also for the pharmacokinetics of the drug, particularly in oral absorption *(97,98)* and central nervous system distribution *(99,100)*. Germline polymorphisms in P-glycoprotein are thus likely to be important for etoposide disposition.

In addition, the pharmacologic effect of etoposide is a function of its interaction with gene products related to the disposition of the drugs with which it interacts. Thus, inactivating polymorphisms in thiopurine methyltransferase result in high levels of thioguanine nucleotide active metabolites, predisposing patients to t-AML *(48)*. Other as yet undiscovered common genetic polymorphisms are also likely to play a role in determining host-risk factors for t-AML as well as the more common toxicities and therapeutic failures attributed to etoposide.

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32 Etoposide and Teniposide

Clinical Applications with a Focus on Leukemia

MALCOLM A. SMITH

CONTENTS

1. INTRODUCTION

Etoposide and teniposide are epipodophyllotoxins that inhibit topoisomerase II, a ubiquitous enzyme that is essential for survival and that plays critical roles in DNA metabolism, chromosome organization, and mitosis *(1)*. Etoposide and teniposide act by stabilizing the covalent linkage between DNA and topoisomerase II *(1)*. These agents have been studied in clinical trials for over 25 yr *(2,3)*, but their contribution to the therapy of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in children remains to be clearly elucidated. There are minor distinctions between etoposide and teniposide. The latter agent is more potent in in vitro tests of cytotoxicity, is associated with higher albumin binding, is more lipophilic, and has a slightly longer plasma elimination half-life (approx 8 vs 6 h) *(4–6)*. However, these differences do not correlate with any therapeutic advantage for teniposide over etoposide in vivo *(7)*. Randomized studies have shown that teniposide and etoposide have similar levels of antitumor activity when used at equitoxic doses *(8,9)*, and current regimens for leukemia that utilize an epipodophyllotoxin employ etoposide. Hence, in this chapter, the two agents are discussed under the assumption that they are more or less interchangeable.

2. DEFINING OPTIMAL SCHEDULES FOR ETOPOSIDE AND TENIPOSIDE

Many different schedules of etoposide administration have been studied in patients with cancer. Because of the cell cycle dependency of topoisomerase II expression and the cell cycle dependency of etoposide in preclinical models *(10,11)*, schedules that provide prolonged exposure would be predicted to be most advantageous. Much of what is known about the relationship between etoposide schedule of administration and antitumor activity comes from trials of patients with small cell lung cancer (SCLC) *(12,13)*. Cavalli et al. *(12)* were the first to present evidence that etoposide was more active against SCLC when given daily for 3–5 d than when given weekly. Subsequently, Slevin and colleagues *(13)* convincingly demonstrated that etoposide administered in five daily doses had a much higher response rate (approx 90%) than the same total dose of etoposide administered as a 24-h infusion (approx 10% response rate). Etoposide pharmacokinetics were measured in all patients in this study, and total areas under the concentration-vs-time curves (AUCs) were equivalent with both regimens. However, the time above a serum level of 1 μg/mL was twofold greater in patients receiving etoposide given daily for 5 d, suggesting that duration above a minimum threshold level is critical for antitumor activity. Subsequent randomized trials demonstrated that extending the period of etoposide treatment to 8 d or to 15 d did not improve the response rate or survival compared with the 5-d schedule *(14,15)*. There are also clinical data from adults with non-small cell lung cancer indicating that teniposide, like etoposide, is more effective when given over several days rather than on a single day *(16)*.

Prolonged administration of oral etoposide has been extensively studied for a variety of solid tumors in adults *(17)*, and

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it has also been evaluated in phase I and II settings in children *(18–21)*. Although antitumor activity has been demonstrated against a number of tumor types and occasional patients considered refractory to etoposide administered on a 3–5 d schedule have responded to etoposide on a prolonged oral administration schedule, there are no convincing data supporting an advantage for prolonged oral administration compared with the schedule of 3–5 d administered intravenously. A randomized trial of etoposide given for either 3 d or 21 d (both in combination with cisplatin) to patients with SCLC showed no benefit for the longer duration of treatment *(22)*. In children, a variant schedule providing prolonged exposure has been evaluated in the phase II setting (daily × 3 administration, repeated weekly for 3 wk). It yielded a surprisingly high response rate of 46% among children with refractory rhabdomyosarcoma *(23)*.

In summary, etoposide shows schedule dependency in both the preclinical and clinical settings. Multiple schedules of administration have been studied with a focus on providing prolonged exposure exceeding the presumed threshold serum levels required for antitumor activity. However, no schedules have proved superior to daily etoposide administration for 5 d.

3. COMBINATIONS OF EPIPODOPHYLLOTOXINS WITH OTHER CYTOTOXIC AGENTS

3.1. Preclinical Rationale and Supporting Clinical Experience

Etoposide and teniposide have been studied in preclinical models in combination with a number of cytotoxic agents. For example, at equitoxic doses in the L1210 murine leukemia model, the combination of etoposide and cyclophosphamide cured 75% of animals compared with <5% for either agent given singly *(24)*. Synergistic interactions between cyclophosphamide and etoposide have also been demonstrated for pediatric solid tumors in xenograft model systems *(25)*. In vitro cytotoxicity data also support favorable interactions when alkylating agents are combined with etoposide. For example, both 4-hydroperoxycyclophosphamide (4-HC) and mafosfamide given with etoposide produced synergistic cytotoxicity in experimental systems modeling purging of AML cells by cytotoxic agents *(26–28)*. Although not commonly used for leukemia treatment, cisplatin and etoposide also showed synergistic activity in in vitro tumor models *(29)*.

The best clinical experience in children evaluating the synergistic activity of alkylating agents combined with epipodophyllotoxins has been obtained for the ifosfamide and etoposide combination, with each agent generally given daily for 5 d. The ifosfamide and etoposide combination is very active against pediatric solid tumors such as Ewing's sarcoma and rhabdomyosarcoma *(30–32)*, and its incorporation into a regimen for children with newly diagnosed Ewing's sarcoma improved both event-free and overall survival *(33)*. The combination of cyclophosphamide and etoposide (each generally given daily × 3–5) also has high levels of activity against pediatric tumors *(34,35)* and has an advantage over the ifosfamide/etoposide combination in terms of decreased cost and diminished risk of nephrotoxicity *(36)*.

The combination of teniposide and cytarabine also demonstrated synergistic activity against the L1210 murine leukemia cell line *(37)*. Clinical studies utilizing this combination are described in the following sections describing the use of epipodophyllotoxins for ALL, AML, and mature B-cell ALL.

3.2. Etoposide and Teniposide for ALL

Etoposide and/or teniposide have been used as a component of therapy for children with newly diagnosed ALL by researchers from St. Jude Children's Research Hospital (SJCRH), the Pediatric Oncology Group (POG), the Medical Research Council (MRC) of the United Kingdom, and others. Examples of epipodophyllotoxin-containing treatment blocks used for ALL are shown in Table 1.

Evaluations of teniposide and etoposide for children with ALL had their genesis in the mid-1970s. SJCRH researchers conducted studies of children with refractory leukemia who received either teniposide or etoposide twice weekly at a dose of 50–100 mg/m^2 *(38)*. Complete responses (CRs) were not observed in any of the 17 children with ALL, although substantial reductions in peripheral blood or bone marrow blast count were observed in five patients *(38)*. When evaluated as a single agent in children with ALL, on a daily × 5 schedule of administration, etoposide produced a single partial response observed among 12 patients *(39)*. Results from these and other published studies suggest that the single-agent activity of either teniposide or etoposide given at conventional doses is limited against advanced recurrent ALL *(40)*.

Subsequent combination studies of teniposide with cytarabine were based on preclinical data showing synergistic activity of these two agents in the murine L1210 model system *(37)*. Clinical evaluations of this combination conducted at SJCRH used each agent administered twice weekly for 4 wk, with response rates of 27 and 64% observed for children with relapsed and refractory ALL, respectively *(41,42)*. The teniposide/cytarabine combination was first used in the treatment of newly diagnosed patients in the Total X study at SJCRH, in which it was given for 2 wk before and 2 wk after standard three-drug remission induction therapy and was also administered five times as 2-wk treatment blocks during the first year of continuation treatment *(43)*. For the 101 children with high-risk features who received therapy with teniposide/cytarabine, the event-free survival (EFS) rate at 9 yr was 36% *(44)*, indicating that teniposide/cytarabine is not sufficient as the sole means of treatment intensification for patients with high-risk ALL *(44)*.

In the SJCRH Total XI study, teniposide/cytarabine was maintained as a component of early treatment, and two courses of high-dose methotrexate were added immediately following induction therapy *(45)*. Other alternating treatment blocks were added as postremission intensification, including etoposide/cyclophosphamide, teniposide/cytarabine, prednisone/vincristine, and conventional-dose methotrexate/mercaptopurine. The clinical outcome in this trial was more favorable than in previous SJCRH studies, with an overall 5-yr EFS rate of 72% *(3)*. This improvement over previous SJCRH studies could have been the result of the intensive use of epipodophyllotoxins but could also have been caused by early intensification with high-dose methotrexate and improved supportive care measures, among other factors. As discussed in a subsequent section,

Table 1
Treatment Blocks Incorporating Etoposide or Teniposide in Pediatric ALL Regimens

Trial	*Drugs*	*Dosage (mg/m²/dose)*	*Day(s)*
MRC UK ALLX (given at wk 5 and 20 of therapy) *(47,48)*	Vincristine	1.5	1
	Daunomycin	45	1–2
	Etoposide	100	1–5
	Cytarabine	100 mg/m²/dose, 12 h	1–5
	Thioguanine	80	1–5
POG ALinC-15 High Risk (given at wk 7, 17, and 27 of therapy) *(50,51)*	Teniposide	165	1–2
	Cytarabine	150	1–3
St. Jude Children's Research Hospital (repeated weekly × 2–4) as evaluated in phase II study and in Total X *(42)*	Teniposide	165	1, 4
	Cytarabine	300	1, 4
Total XI postremission intensification rotating pairs (epipodophyllotoxin-containing drug pairs given either weekly × 6 or given every 4 wk, alternating with methotrexate/6-mercaptopurine and with vincristine/prednisone drug pairs) *(45)*	Teniposide	150	1
	Cytarabine	300	1
	Etoposide	300	1
	Cyclophosphamide	300	1
Total XII postremission intensification (given every 12 wk) *(46)*	Teniposide	200	1
	Cytarabine	300	1
POG infant ALL pilot study *(54)*	Etoposide	100	1–5
	Cyclophosphamide	300	1–5

Abbreviations: MRC, Medical Research Council; POG, Pediatric Oncology Group.

concerns about the excessive risk of secondary AML first arose in the Total X and Total XI studies.

More recent studies of ALL at SJCRH have continued to assess teniposide as a component of both induction therapy and postremission intensification therapy, with the Total XII study using teniposide/cytarabine five times in the first year following the achievement of remission *(46)*. The dose of teniposide for one-half of the patients was individualized based on the patients' rates of drug clearance, a maneuver that did not appear to be associated with improved outcome compared with the conventional approach of basing teniposide dose on body surface area *(46)*.

Both the MRC and POG have conducted randomized trials demonstrating that postremission treatment intensification with regimens that include etoposide is associated with improved outcome. However, in those randomized studies, the multiple other agents in the superior treatment regimen in addition to etoposide make identification of a specific etoposide contribution impossible. In the MRC UK ALLX trial, for example, children with newly diagnosed ALL were randomized to receive intensification therapy with an etoposide-containing treatment block at 5 wk, 20 wk, both, or neither *(47,48)*. Children who received the treatment block twice had significantly superior 5-yr disease-free survival (DFS; 70–71%), compared with children who received the treatment block only once (61–62%), or not at all (56%) *(47,48)*. The contribution of etoposide as one of five drugs in this multiagent intensification regimen cannot be isolated, and the successor randomized studies (UK ALLXI and MRC ALL97) demonstrated that further intensification with a non-etoposide-containing treatment block at wk 35 significantly improved outcome when added to a regimen including intensification with the etoposide-containing treatment blocks given at wk 5 and 20 *(49)*.

POG investigators have used teniposide in combination with cytarabine for children with high-risk ALL *(50,51)*. The POG ALinC-15 study for children with high-risk ALL randomized patients between a regimen utilizing intensive intermediate-dose methotrexate given with intravenous mercaptopurine and a regimen utilizing intensive alternating chemotherapy (including the teniposide/cytarabine treatment block and an anthracycline/cytarabine/asparaginase-containing treatment block). Children receiving the intensive alternating chemotherapy that included teniposide had significantly higher 2-yr EFS rates than children receiving the regimen utilizing only antimetabolites (84% vs 75%, $p = 0.006$).

Etoposide has also been used in combination with cyclophosphamide or ifosfamide for the treatment of ALL in children, based on the preclinical data described previously concerning the synergistic activity of alkylating agents combined with etoposide. Using the same schedule of administration of ifosfamide and etoposide that has been shown to improve outcome for children with Ewing's sarcoma (daily × 5 for both agents) *(33)*, different groups of investigators obtained complete responses in 30–40% of patients with relapsed ALL *(52,53)*. A treatment block using cyclophosphamide and etoposide (both given daily × 5) is a component of an intensive chemotherapy regimen for infant ALL that has shown promising disease control in its early evaluation *(54)*.

3.3. Etoposide and Teniposide for AML

Etoposide has most commonly been studied as a single agent for AML administered on a daily × 5 schedule with doses rang-

Table 2
Treatment Blocks Incorporating Etoposide in Pediatric AML Regimens

Trial	*Drugs*	*Dosage (mg/m²/dose)*	*Day(s)*
CCG intensive-timing DCTER regimen *(68)*	Dexamethasone	6	0–3, 10–13
	Cytarabine	200 (CI)	0–3, 10–13
	Thioguanine	100	0–3, 10–13
	Etoposide	100 (CI)	0–3, 10–13
	Daunomycin (rubidomycin)	20 (CI)	0–3, 10–13
Australian Leukemia Study Group *(74)*	Cytarabine	100 (CI)	1–7
	Daunomycin	50	1–3
	Etoposide	75	1–7
POG mitoxantrone, etoposide ± cyclosporine treatment block *(67)*	Mitoxantrone	10	1–5
	Etoposide	100	1–5
	or		
	Mitoxantrone	6	1–5
	Etoposide	60	1–5
	Cyclosporine (CSA)	CI × 100 h (CSA levels > 2400 ng/mL)	
MRC AML10 ADE treatment block *(72)*	Cytarabine	100 mg/m²/dose, q12h	1–10
	Daunomycin	50	1, 3, 5
	Etoposide	100	1–5
AML BFM-87 regimen *(97)*	Cytarabine	100 (CI)	1–2
	Cytarabine	100 mg/m²/dose, q12h	3–8
	Daunomycin	30 mg/m²/dose, q12h	3–5
	Etoposide	150	6–8

Abbreviations: CI, continuous infusion; CCG, Children's Cancer Group; POG, Pediatric Oncology Group; MRC, Medical Research Council; BFM, Berlin–Frankfurt–Münster Group.

ing from 50 to 200 mg/m²/d, as summarized by Stadtmauer et al. *(40)*. Etoposide has clear activity against AML, producing CRs in approx 17% of patients *(55)*. An analysis of response to single-agent etoposide by French-American-British (FAB) classification subtype suggested that responses were more frequent for FAB M4 and M5 cases (approx 30%) compared with M1 and M² cases (approx 6%) *(40)*, with several reports documenting activity for etoposide in children with M4 and M5 FAB subtypes *(56,57)*. Etoposide (or in some cases teniposide) has been combined with many agents to treat patients with AML, including cytarabine *(58)*, amsacrine *(59–61)*, 5-azacytidine *(62,63)*, cyclophosphamide *(64,65)*, and mitoxantrone *(66,67)*. Table 2 lists examples of currently utilized treatment blocks for AML therapy that include etoposide.

Etoposide has been used in Children's Cancer Group (CCG) protocols for children with AML as part of a five-drug induction therapy regimen given in 4-d blocks, which in addition to etoposide includes cytarabine, dexamethasone, thioguanine, and daunomycin (the DCTER regimen). In the CCG-213 study, this five-drug regimen produced a 76% CR rate, and children entered onto the study had a 3-yr EFS of 35% *(68)*. This five-drug combination was utilized in the CCG-2891 study, in which patients were randomized to receive the second DCTER cycle either 10 d after the first cycle began (intensive timing), or 14 d or later from the beginning of the first cycle, depending on the bone marrow status (standard timing) *(69)*. Patients randomized to the intensive timing arm had a significantly better outcome than patients randomized to the standard timing arm (3-yr, 42% vs 27%). However, the CCG-2891 study did not address whether "intensive timing" with 8 d of treatment interrupted at the midpoint by a 6-d rest is superior to 8 consecutive days of treatment with these drugs at similar doses. Data from other large clinical trials for childhood AML suggest that similar outcomes are obtained with etoposide-containing regimens that use 8 or 10 consecutive days of therapy *(70–73)*.

Etoposide has been used with mitoxantrone for children with relapsed and/or refractory AML, with cyclosporine added in an attempt to improve outcome by reversing multidrug resistance *(67)*. The use of cyclosporine at doses sufficient to achieve serum levels associated with P-glycoprotein modulation resulted in reduced hepatic clearance of both etoposide and mitoxantrone, so the dosages of each had to be reduced by approx imately 40% in order to be equitoxic with the drugs used without cyclosporine. The CR rate with this three-drug combination was 35% *(67)*, and POG investigators built on this experience to develop a randomized trial (POG-9421) to evaluate whether cyclosporine improved outcome when given with mitoxantrone/etoposide as part of AML consolidation therapy.

In contrast to the situation for ALL, randomized studies have addressed the contribution of etoposide to regimens used to treat newly diagnosed patients with AML. The Australian Leukemia Study Group conducted a randomized trial in 264 adults with AML that evaluated the contribution of etoposide when added to standard dose cytarabine and daunomycin during induction

Table 3
Treatment Blocks Incorporating Etoposide or Teniposide in Mature B-Cell ALL Regimens

Trial	*Drugs*	*Dosage (mg/m²/dose)*	*Day(s)*
NHL-BFM 86 *(81)*	Dexamethasone	10	1–5
	Ifosfamide	800	1–5
	Methotrexate	5 g/m², 24-h CI	1
	Cytarabine	150 mg/m² q12h	4–5
	Etoposide	100	4–5
	Vincristine	1.5	1
LMB CYVE treatment block *(76,77)*	Cytarabine	50 mg/m² as 12-h infusion	1–5
	Cytarabine	3 g/m²/d	2–5
	Etoposide	200	2–5
NCI Pediatric Branch IVAC treatment block *(80)*	Ifosfamide	1500	1–5
	Cytarabine	2 g/m² q12 h	1–2
	Etoposide	60	1–5
POG ifosfamide/etoposide treatment block *(82)*	Etoposide	100	1–5
	Ifosfamide	2800	1–5

Abbreviation: CI, continuous infusion; NHL, non-Hodgkin's lymphoma; BFM, Berlin–Frankfurt–Münster Group; NCI, National Cancer Institute; POG, Pediatric Oncology Group.

and consolidation therapy *(74)*. The addition of etoposide significantly improved remission duration for the overall patient population (18 mo vs 12 mo, $p = 0.01$), and subset analysis suggested that in patients younger than 55 yr etoposide significantly prolonged both remission duration (27 mo vs 12 mo, $p = 0.01$) and survival (17 mo vs 9 mo, $p = 0.03$).

The contribution of etoposide to induction therapy for children and adults with AML was evaluated in the MRC AML10 trial *(75)*, in which 1857 eligible patients (286 children younger than 15 yr) were randomized between two three-drug regimens: DAT (daunomycin, cytarabine, thioguanine) and ADE (cytarabine, daunomycin, etoposide). For both the overall patient population and the subset of children younger than 15 yr, there were no significant differences between the two induction treatments in CR rate and in DFS and overall survival at 6 yr. For children younger than 15 yr, the CR rate was 89.5% with DAT and 93.0% with ADE ($p = 0.30$) *(72)*. There was also no significant difference between the DAT and ADE regimens in longer term measures of efficacy: EFS at 7 yr was 48% for DAT and 45% for ADE; survival at 7 yr was 58% for DAT and 50% for ADE ($p = 0.20$); DFS at 7 yr from CR was 53% for DAT and 48% for ADE ($p = 0.30$). Subgroup analysis failed to suggest any benefit for etoposide in patients with FAB type M4 or M5, although the numbers of patients in these subsets limited the ability to identify differences *(72)*.

3.4. Etoposide and Teniposide for Mature B-Cell ALL

Epipodophyllotoxins are employed in several multiagent regimens used for the treatment of mature B-cell ALL (Table 3). Etoposide given for 4 consecutive d in combination with high-dose cytarabine (also given for 4 d) was shown to be active against relapsed or refractory B-lineage non-Hodgkin's lymphoma (NHL), with 8 of 12 patients showing objective responses *(76)*. This regimen (termed CYVE) was incorporated into the LMB-86 and LMB-89 protocols of the French Society of Pediatric Oncology (SFOP), with 88% EFS rates observed in the LMB-89 study for children with stage IV NHL and mature B-cell ALL *(76,77)*.

The Pediatric Branch of the National Cancer Institute (NCI) developed a regimen (termed IVAC) that added high-dose cytarabine (four doses given over 2 d) to ifosfamide and etoposide (each given daily × 5). This three-drug regimen was very myelotoxic but had high levels of activity in patients with recurrent NHL *(78)*. When it was incorporated into a regimen for newly diagnosed patients, an EFS rate of 75–80% was observed for patients with stage IV NHL and mature B-cell ALL *(79,80)*.

Other groups have also incorporated epipodophyllotoxins into treatment protocols for mature B-cell ALL and B-lineage NHL *(81,82)*. POG researchers evaluated the ifosfamide and etoposide combination for this patient population and observed that patients with CNS disease who had ifosfamide/etoposide added to standard therapy appeared to have significantly improved outcome in comparison with historical controls *(82)*.

4. SECONDARY LEUKEMIA RISK FROM EPIPODOPHYLLOTOXINS

A significant concern about the use of etoposide and teniposide is their association with treatment-related leukemia. Such leukemias characteristically have a short latency period, a FAB M4 or M5 subtype, and rearrangements of the *MLL* gene at chromosome 11q23 *(83)*. However, other types of leukemia may also follow treatment with regimens containing epipodophyllotoxins *(84)*, and molecular abnormalities other than *MLL* gene rearrangements can be observed following treatment with epipodophyllotoxins *(85)*.

Of primary clinical interest in decision making about the use of etoposide or teniposide in chemotherapy regimens is the quantitative risk of developing secondary leukemia following epipodophyllotoxin treatment, as well as the relationship

between epipodophyllotoxin cumulative dose and schedule and the risk of subsequent secondary leukemia. Unacceptably high estimates of the cumulative risk for leukemia (5% to >10%) have been reported for children with ALL and lymphoblastic lymphoma treated with epipodophyllotoxins using schedules of administration other than the daily × 5-d schedule *(86–89)*. These high estimates differ from the risk of secondary leukemia for patients receiving epipodophyllotoxins at lower cumulative doses administered on a daily × 3 or daily × 5 schedule. For example, estimates of risk for patients with germ cell tumors receiving etoposide (daily × 5 schedule to a cumulative dose of ≤ 2.0 g/m^2) in combination with cisplatin and bleomycin are approx imately 0.5% *(90)*. In the UK ALLXI and MRC ALL97 studies in which etoposide was given on a daily × 5 schedule to cumulative doses ranging from 0.5 to 1 g/m^2, only two cases of secondary AML were observed among 729 patients *(49)*. Other regimens for children with ALL that utilized low cumulative dosages of etoposide also carried a low risk of secondary leukemia *(84)*, similar to that for regimens not containing an epipodophyllotoxin *(91–93)*.

As the epipodophyllotoxins are cell cycle-specific agents that produce widely disparate antitumor effects when given on different schedules (see preceding discussion), it is plausible that schedule may play an important role in determining leukemia risk. There are in vitro data supporting an increased leukemia risk for intermittent exposure schedules modeled after those used in leukemia regimens *(94,95)*, and clinical results suggest increased leukemogenicity for some intermittent schedules of administration *(87,96)*. The etoposide administration schedule associated with the highest cumulative incidence of secondary leukemia was weekly or twice-weekly administration *(87,88,96)*.

5. SUMMARY

The contributions of etoposide and/or teniposide to the successful treatment of pediatric leukemias are not well defined. The development of these agents for the leukemias was influenced by preclinical studies showing favorable interactions in combination with alkylating agents and cytarabine and by preclinical and clinical observations showing the importance of prolonged exposure above a threshold level. Most etoposide-containing regimens currently used for the treatment of childhood leukemias use a daily × 3–5 schedule of administration in combination with cytarabine and/or an alkylating agent. As there has been only one randomized study for children with leukemia that isolated the contribution of etoposide, the summary comments that follow regarding the role of etoposide and/or teniposide are essentially observational.

For childhood ALL, it is clear from studies of the CCG and the Berlin–Frankfurt–Münster (BFM) group that outcome similar to that obtained with epipodophyllotoxin-containing regimens can be achieved with regimens that do not include epipodophyllotoxins. A nationwide clinical trial in the United States for infants with newly diagnosed ALL combined etoposide with cyclophosphamide (both given daily × 5) as a component of postremission intensification therapy *(54)*. Definitively establishing a role for the cyclophosphamide/etoposide combination for infant ALL or for another high-risk ALL population would require an appropriately designed randomized clinical trial. The twice-weekly schedule of cytarabine and teniposide that demonstrated activity against relapsed ALL has subsequently been shown to be associated with a high risk of secondary AML, which has eliminated enthusiasm for use of this schedule.

For pediatric AML, etoposide is a component of induction therapy for the MRC and BFM regimens that have produced the highest EFS rates published for childhood AML. A single randomized trial in adults has shown an advantage in terms of EFS, but not survival, for the inclusion of etoposide with cytarabine and daunomycin during induction therapy. Another randomized trial showed etoposide to be equivalent to thioguanine as a third agent added to cytarabine and daunomycin. Early reports of a specific role of etoposide in the treatment of AML with monocytic differentiation have not been confirmed in larger randomized trials.

For mature B-cell ALL and advanced-stage B-lineage NHL, etoposide is a component of the effective LMB-86 and LMB-89 regimens developed and studied by the SFOP. Etoposide-containing regimens have also been studied by the POG and the Pediatric Branch of the NCI. Historical comparisons suggest that incorporation of these etoposide-containing combinations into treatment programs for mature B-cell ALL improved outcome, although definitive evidence for their contribution would require a properly designed randomized clinical trial.

Concerns over an excessive risk of secondary AML have been substantially allayed for etoposide given on a daily × 3–5 schedule to moderate cumulative doses (<2000 mg/m^2). Experience in thousands of patients receiving etoposide via this schedule has demonstrated that the cumulative risk of secondary AML is unlikely to be >1%. Furthermore, additional experience with dose-intensive chemotherapy regimens not including etoposide or teniposide have demonstrated that the epipodophyllotoxins are by no means unique in inducing secondary leukemias.

Although etoposide and teniposide may be maintained in the future as components of some regimens for the treatment of childhood ALL and AML, it is unlikely that manipulations involving these agents will substantially improve outcome for the 50–60% of children with AML and the approx 20% of children with ALL who are not cured with current treatment strategies. For these patients, new therapeutic approaches building on increased understanding of the cellular pathways involved in leukemia cell growth and survival are more likely to be successful.

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IV

Hematopoietic Stem Cell Transplantation

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Perspective 1

FREDERICK R. APPELBAUM

CONTENTS

1. INTRODUCTION

The application of hematopoietic stem cell transplantation to the treatment of acute leukemia is worth considering for at least two reasons. First, although transplantation is widely used as treatment for acute leukemia, its exact role requires reexamination because of recent advances in both transplantation and chemotherapy as well as the development of better prognostic markers. Second, the experience gained with transplantation continues to provide insights into the behavior of acute leukemia and its response to therapies that may prove to be more broadly applicable. In this chapter, the current applications and outcomes of hematopoietic stem cell transplantation for the treatment of acute leukemia are reviewed and several areas of current research interest are discussed.

2. CURRENT INDICATIONS AND OUTCOMES

2.1. Allogeneic Transplantation from Matched Siblings

2.1.1. Acute Myeloid Leukemia

For patients who fail primary induction chemotherapy, allogeneic transplantation is the only treatment that offers any hope for cure, with two studies reporting a 15–20% success rate in such patients *(1)*. Since it takes time to identify a donor and arrange for the transplant, patients and their families should be HLA-typed soon after diagnosis to facilitate a transplant, should induction therapy fail. Patients transplanted from matched siblings in second remission have a 35–40% probability of survival at 5 yr from transplant, results that are generally acknowledged to be superior to what can be achieved with chemotherapy *(2)*. Given that patients transplanted for more advanced disease do worse, there would appear to be little reason to delay transplantation beyond second remission for patients with acute myeloid leukemia (AML) and a matched sibling. Several limited studies suggest that transplantation in untreated first relapse yields results almost as good as those achieved with transplantation in second remission *(3,4)*. Since some patients may die during attempted reinduction and others develop toxicities during the attempt that could preclude subsequent transplant, the question of the need for reinduction prior to transplantation has been raised. Although the question has never been studied in a prospective randomized fashion, retrospective data have led most experts to recommend considering immediate transplantation if relapse is detected early and the transplant can be initiated before the patient has circulating blasts. Otherwise, reinduction is generally recommended.

The best results with allogeneic transplantation have been obtained when it is carried out in first remission. At least 15, and probably more, prospective trials have been conducted in which patients with matched siblings have been assigned to allogeneic transplantation whereas those without donors have been treated with consolidation chemotherapy (reviewed in ref. *5)*. Among these 15 trials, the 3-yr disease-free survival with transplantation has varied between 40 and 64%; chemotherapy has yielded 3-yr disease-free survival rates of 19–24%. These studies, although useful in providing general estimates

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of expected outcome, are not very helpful in determining the best approach for any individual patient, for several reasons. First, because such studies take so long to conceive, conduct, and report, they may not reflect current technologies. Second, they all have compared the outcome of transplantation with that with chemotherapy, but for the patient with a donor, the question of interest is really transplantation in first remission verus delaying transplantation until first relapse. Finally, these studies examined the general effects of these two interventions without considering relative effects among various subgroups of patients.

More recently, several trials have examined the relative effects of matched sibling transplantation for AML in first remission, compared with intensive consolidation according to cytogenetic risk group. An Eastern Cooperative Oncology Group-Southwest Oncology Group (ECOG-SWOG) intergroup study found a relative benefit for allogeneic transplant compared with chemotherapy for patients with leukemia assigned to the favorable-risk group and for those in the unfavorable risk group, but equivalent outcomes for patients with an intermediate risk of relapse *(6)*. Other factors, such as patient age and cytomegalovirus serostatus, also impact the outcome of transplantation, whereas factors such as the white blood cell count at diagnosis and multidrug resistance phenotype may influence the outcome of chemotherapy. No single study comparing transplantation with chemotherapy is likely to be large enough to be informative about all of these factors, so that clinicians must, to a considerable degree, rely on the aggregate results of multiple trials together with their clinical judgment. At present, for patients with matched siblings, transplantation in first remission can be strongly recommended for patients with unfavorable cytogenetic features. It is a reasonable approach for those with intermediate- or good-risk disease, particularly if they are younger and in good health. For older patients or those with medical problems that could complicate the transplant procedure, an approach of withholding transplantation until first relapse is not unreasonable.

2.1.1. Acute Lymphoblastic Leukemia

The role of matched sibling transplantation for adults with acute lymphoblastic leukemia (ALL) in many ways parallels that for AML, although fewer studies have been performed, reflecting the lower incidence of the disease. Allogeneic transplantation is the only form of therapy able to cure patients who have failed primary induction therapy, curing 15–20% of such patients *(1)*. Approximately 30% of adults with ALL in second remission can be cured with matched sibling transplantation, a result superior to anything published on alternative therapies *(7,8)*. As in AML, clinicians are sometimes faced with the question of whether to reinduce patients with ALL in first relapse before proceeding to transplantation.

There are scant published data to help inform this choice, but a reasonable approach would be to attempt reinduction in most patients except those in very early relapse or with a very short initial remission, who would be felt to be unlikely to respond completely to chemotherapy. A number of pilot studies of matched sibling transplantation for ALL in first remission have been published with encouraging results, but few large prospective trials have been reported. The French Group for Therapy of Adult ALL conducted a study in which patients with matched siblings were allocated to allogeneic transplantation whereas those without were randomized between consolidation chemotherapy and autologous transplantation *(9,10)*. There was no difference in 5-yr overall survival between the autologous transplant and chemotherapy groups, with a combined survival of 35% for these two populations. In contrast, the 5-yr survival of the allogeneic group was 48%. The relative advantage of allogeneic transplantation was more marked among high-risk patients, defined as those with Philadelphia chromosome-positive (Ph+) leukemia, age > 35 yr, initial white blood cell count in excess of 30×10^9/L, or prolonged time (>4 wk) to attain initial complete remission (CR). Although this study suggests a definite advantage for allogeneic transplantation in first remission for high-risk patients, it is subject to some of the same criticisms made of AML studies, including a failure to account for the impact of salvage transplantation at first relapse, progress in both modalities since these studies were initiated, and insufficient power to discern different effects among smaller subgroups of patients.

Because chemotherapy regimens for ALL are far more effective in children than adults, the indications for matched sibling transplantation differ. Although transplantation is clearly indicated for patients who fail primary induction, the indications for patients in second remission have been more debatable. Recently, the results of allogeneic transplantation in 376 children in second CR were compared with those of 540 children treated with chemotherapy *(11)*. The probability of disease-free survival at 5 yr was higher after transplantation ($40 \pm 3\%$ vs $17 \pm 3\%$, $p < 0.001$), and the relative benefits of transplantation were seen in both high-risk and lower-risk patients. The expected outcome of chemotherapy for the large majority of pediatric patients in first remission are excellent, eliminating any need for the more toxic allogeneic transplant in such patients. However, there are several very high-risk groups, such as those with leukemia characterized by Ph positivity or the t(4;11), in whom retrospective results suggest an advantage for transplantation.

2.2. Allogeneic Transplantation from Alternative Donors

Only approximately one in three patients will have a matched sibling donor, so alternative donors are needed for most patients who require a transplant. The outcome of transplantation from family members mismatched with the patient for a single antigen yields survival very close to that seen with fully matched siblings, albeit with a somewhat higher incidence of graft vs host disease (GvHD) but a compensatory drop in relapse rates. In the experience of most investigators, transplantation from family member donors mismatched for two or more antigens has been associated with a high rate of GvHD, graft failure, and other complications, leading to markedly worse survival. Although some limited experiments have reported encouraging results using, for example, vigorous T-cell depletion plus addition of peripheral CD34+ cells or attempts at inducing tolerance by in vitro manipulation pretransplant, much more follow-up is required to make judgments about these approaches *(12,13)*.

With the formation of the National Marrow Donor Program (NMDP) and the typing of more than 4 million volunteer donors, it is now possible to find donors for 75% of patients for whom a search is initiated. Although results may vary among transplant centers, overall survival after unrelated donor transplantation has generally been slightly worse than survival following matched sibling transplantation. For example, the International Bone Marrow Transplant Registry (IBMTR) reports a 4-yr survival rate of 50% with unrelated donors vs 60% for matched sibling donors for transplantation for AML in first remission. Similarly, the 4-yr survival for AML in second remission is approximately 35% with unrelated donors vs 40% with matched siblings. The magnitudes of differences in outcome in ALL are similar. The major reason for the somewhat poorer results following unrelated donor transplantation is an increase in GvHD and infectious complications related to its treatment. A multivariate analysis examining features associated with improved survival following unrelated donor transplantation for acute leukemia was conducted in Seattle *(14)*. Not unexpectedly, undergoing transplantation in remission and being cytomegalovirus seronegative were both associated with an improved outcome. In addition, receiving a higher marrow cell dose per kilogram of body weight was also associated with an improved outcome, although the exact reason for this association is less clear. The beneficial effect appears to be independent of age. Recipients of high marrow cell doses appear to engraft more rapidly and to have less GvHD and a lower transplant-related mortality rate.

2.3. Autologous Marrow Transplantation

A large number of phase II studies exploring the application of autologous transplantation for the treatment of AML in first and second remission have been published (reviewed in ref. *15*) By comparison with results seen following matched sibling transplantation, these studies have reported higher relapse rates but lower rates of transplant-related mortality. The end results have been survivals similar to or slightly worse than those obtained with allogeneic transplantation. No prospective randomized trials have evaluated the role of autologous transplantation vs aggressive chemotherapy for patients with AML in second remission. There have, however, been several studies evaluating the use of autologous transplantation in first remission.

The European Organization for Research and Treatment of Cancer (EORTC) evaluated allogeneic transplantation for patients with matched siblings vs autologous transplantation vs intensive chemotherapy ($n = 333$) *(16)*. Disease-free survival at 4 yr was 54% with allogeneic transplantation vs 48% with autologous transplantation vs 30% with continued chemotherapy. The Medical Research Council of the United Kingdom randomized 381 patients who had received three cycles of consolidation chemotherapy to no further therapy or to autologous transplantation *(17)*. Those randomized to the transplant group had a significantly lower incidence of recurrent disease and improved disease-free survival at 7 yr (53% vs 40%). Finally, an American Intergroup Study found no overall benefit of autologous transplantation when patients were randomized after induction and a single cycle of consolidation of modest intensity to receive either high-dose cytarabine or an autologous transplant. Taken in aggregate, these studies suggest that autologous transplantation does not appear to improve outcome when it is used as a substitute for intensive consolidation, but it may improve the likelihood of cure when used in addition to intensive consolidation.

There are fewer data concerning the role of autologous transplantation in the treatment of ALL. As in AML, relapse rates appear higher, but transplant-related mortality rates seem to be lower after autologous transplantation than with allogeneic transplantation. The IBMTR reports 3-yr disease-free survivals of 47% with 139 patients transplanted in first CR, 35% for 227 patients transplanted in second CR, and 14% for 33 patients done in relapse. In the only prospective examination of allogeneic transplantation vs autologous transplantation vs conventional chemotherapy for adult ALL in first remission, referred to earlier, no advantage was found for autologous transplantation compared with chemotherapy *(9,10)*.

3. AREAS OF CURRENT RESEARCH INTEREST

3.1. Source of Stem Cell for Allogeneic Transplantation

3.1.1. Bone Marrow vs Mobilized Peripheral Blood

As will be discussed, results following autologous transplantation demonstrated that the use of growth factor-mobilized peripheral blood stem cells led to faster engraftment than seen with the use of unmanipulated bone marrow. There was initial hesitation to study peripheral blood stem cells in the allogeneic setting because such products contain many more T-cells than marrow, and in animal models the extent of GvHD is related to the number of T-cells included in the stem cell product. A series of early pilot studies of the use of granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells for transplantation in HLA-identical siblings suggested, however, that their use was not necessarily associated with an increase in acute GvHD but did, as expected, lead to more rapid engraftment *(18–20)*. The conclusions from these first patients were in part supported by broader phase II trials that confirmed more rapid engraftment and no striking increase in the incidence or severity of acute GvHD. These studies, however, were inconsistent with regard to chronic GvHD, some showing an increase in incidence with peripheral blood stem cells and others finding no difference.

Recently, a large prospective phase III randomized trial comparing G-CSF-mobilized peripheral blood stem cells with unmanipulated bone marrow for transplantation of matched siblings has been completed *(21)*. In this trial, 177 patients with hematologic malignancies were randomized to either source of stem cells; all patients received the same form of GvHD prophylaxis (methotrexate plus cyclosporine) and the same forms of supportive care. As anticipated, patients receiving peripheral blood engrafted faster, with recovery of granulocyte and platelet function approx 1 wk in advance of those receiving bone marrow. There was no significant difference in acute or chronic GvHD. An unexpected outcome of the study was that the overall survival as well as disease-free survival was significantly improved in the group receiving peripheral blood stem cells. This advantage was more marked in those with more advanced hematologic malignancies.

Although recipients of peripheral blood tended to have a lower incidence of disease recurrence and death from idiopathic interstitial pneumonia, no one cause of treatment failure accounted for the poorer outcome seen with marrow. These results suggest that growth factor-mobilized peripheral blood should be considered the standard of care of patients undergoing transplantation from matched siblings, particularly if they have more advanced leukemia.

The reasons why G-CSF-mobilized peripheral blood stem cells were not associated with more GvHD are unclear. Some have suggested that the functional characteristics of T-cells collected after G-CSF priming may be altered, with polarization toward Th2 as opposed to Th1 helper cells *(22)*. More recently, it has been shown that G-CSF mobilizes far more type II dendritic cells than are normally found in bone marrow, which in turn favors outgrowth of Th2 T-cells *(23)*. In addition to attempting to understand better the biology of G-CSF-mobilized peripheral blood stem cell products, current studies are asking whether the product can be further manipulated to improve outcome, for example, by limiting the total number of T-cells. Additionally, studies are now under way to test the use of peripheral blood stem cells for unrelated donor transplantation. Such studies are warranted not only by the results in matched siblings, but also by the observation of an association of improved survival in recipients of matched unrelated marrow with higher doses of marrow cell.

3.1.2. Cord Blood Transplantation

Umbilical cord blood contains a relatively high concentration of CD34+ cells that can serve as a source of stem cells for transplantation. In an initial series of 44 children treated with cord blood from siblings, myeloid engraftment occurred in most patients at a pace similar to that seen with marrow, whereas platelet recovery was slower *(24)*. The incidence of acute GvHD was low, reflecting both the young age of the patients and the relative paucity of T-cells within cord blood. Given these results, large-scale banking of cord blood products for subsequent unrelated transplantation was initiated. A summary of the first 272 unrelated cord blood transplants facilitated by the New York Blood Center's Program revealed engraftment in approximately 90% of individuals, but the tempo of engraftment was significantly delayed, particularly for platelets *(25)*. A dose relationship was seen among the number of nucleated cord blood cells infused, the incidence and speed of engraftment, and overall survival. Currently, it remains difficult to find cord blood units with sufficient cell counts to engraft most full-grown adults safely, but methods to expand cord blood products in vitro or facilitate engraftment in vivo are under active study.

3.1.3. Matched Unrelated Donors

Only approximately one-third of patients will have an HLA-identical sibling to serve as a donor for transplantation. Because of the highly polymorphic nature of the HLA antigen, there is little likelihood that any two unrelated individuals will be HLA-matched. However, with the formation of the NMDP in the United States and other such registries around the world, several million normal individuals have been HLA-typed and have agreed to serve as stem cell donors. This has increased the odds of finding a suitably matched unrelated donor to at least 50%. On average, it takes approximately 4 mo from the initiation of a search to identify a donor and begin the transplant procedure. In general, most studies suggest a somewhat higher incidence of GvHD with the use of unrelated donors but a slight decrease in disease recurrence. Overall survival at most centers has been slightly less with the use of unrelated donors compared with matched siblings.

HLA typing for donor selection has generally involved typing at the DNA level for class II antigens but reliance on serotyping for class I antigens. Recent studies show that as many as 30% of unrelated donors serologically identical for class I antigens will have some degree of mismatching at the class I alleles *(26)*. In the setting of transplantation for chronic myeloid leukemia (CML), such mismatching has been associated with an increased incidence of GvHD and with distinctly worse overall survival if two or more such mismatches are seen. Since methods are now available to perform typing at the DNA level for class I antigens, it has become possible to select among multiple donors who appear equally well matched by serologic typing.

3.2. Stem Cells in Autologous Transplantation

The two topics that have dominated discussions of the technology of autologous transplantation in acute leukemia beyond its general place in the overall management of patients are the relative advantages of mobilized peripheral blood vs marrow and the role of purging. A large number of phase II and several fairly small phase III studies, each employing patients with a variety of diseases, demonstrated that the use of mobilized peripheral blood stem cells when compared with unstimulated marrow led to faster engraftment, the need for fewer transfusions, and shorter hospitalizations. Based on these results, use of growth factor-mobilized peripheral blood stem cells has now become standard, and estimates from the IBMTR are that <10% of autologous transplants were done with bone marrow in 1999. Although this switch may be appropriate, it is at least interesting to consider that it has occurred without any large prospective randomized trial examining the outcome of peripheral blood vs marrow in any single disease. A retrospective study from the European Bone Marrow Transplant Group comparing marrow with peripheral blood autografts for AML in first complete remission reported leukemia-free survival of 51% in 1279 recipients of marrow vs 44% in 100 recipients of peripheral blood *(15)*. In this retrospective analysis, improved results were seen when purged marrow was used or when leukopheresis was performed only after patients had received a minimum of two chemotherapy courses.

Using genetic marking, Brenner et al. *(27)* demonstrated that, in some cases, leukemic cells from the autologous marrow inoculum can "contribute" to posttransplant relapse. This result, which confirms what most investigators had assumed to be true, provides a rationale for attempting to eliminate residual tumor cells from the autologous stem cell inoculum. For AML, the largest experience has been with the cyclophosphamide derivative 4-HC and mafosfamide. There have been no large randomized trials evaluating the role of purging in AML, but retrospective analyses of purging have been performed. In these

studies, a relative advantage for purging was seen in some subgroups of patients, particularly in those conditioned with total-body irradiation (TBI) and transplanted within 6 mo of induction *(15)*. Other methods for purging have been developed, including the use of monoclonal antibodies directed at cell surface antigens, such as CD15, expressed by many leukemic progenitors but not on the normal hematopoietic stem cell *(28)*. As in the case of pharmacologic purging, no randomized trials have yet been completed evaluating this approach.

There has been little recent activity in developing purging techniques in ALL in large part because the overall clinical activity in autologous transplantation for this disease has waned. In both AML and ALL, the development of purging technologies has been severely affected by the inability to conduct prospective clinical trials of appropriate size to address the question of the efficacy of the approach. If reliable surrogate markers existed for disease persistence vs elimination, it might be possible to develop more efficient trials that could approach these significant questions.

4. DISEASE ERADICATION

4.1. Preparative Regimens

The intensive preparative regimen given prior to transplantation is designed to eliminate the malignancy and, in the case of allogeneic transplantation, sufficiently immunosuppress the patient to allow engraftment. Despite the central role the preparative regimen plays in transplantation, there have been surprisingly few randomized trials attempting to identify optimal regimens for specific conditions. For patients with AML in first remission, randomized trials have suggested an advantage of fractionated TBI over single-dose TBI and of cyclophosphamide plus fractionated TBI over cyclophosphamide plus busulfan *(29,30)*. In other randomized trials, no advantage was found when melphalan was substituted for cyclophosphamide in TBI-containing regimens, and finally, in a study comparing 12 Gy of fractionated TBI with 15.75 Gy, the higher TBI dose was found to be associated with fewer relapses but a higher rate of transplant-related mortality, resulting in no overall change in survival *(31,32)*. These four studies represent the only randomized trials of preparative regimens for AML in first remission published to date. A retrospective analysis from the European Bone Marrow Transplant Group compared the outcome of cyclophosphamide plus TBI with that seen with cyclophosphamide plus busulfan in 446 patients and found no difference, calling into question the general applicability of the results of the randomized trial referred to earlier *(33)*. There are almost no randomized trials of preparative regimens for the treatment of ALL. Thus, at present, there are few data to aid in the identification of a superior preparative regimen for patients with acute leukemia in any specific disease category.

Other approaches are being developed with encouraging results, but none as yet have been tested in prospective randomized trials. The group from City of Hope has combined VP16 (etoposide) with TBI and has seen long-term disease-free survival in about 60% of patients transplanted for AML in first remission *(34)*. Another approach piloted by the Sloan-Kettering group involves the use of lectin-based T-cell-depleted marrow with a preparative regimen of cyclophosphamide, thiotepa, and fractionated TBI *(35)*. An approach being studied at the Fred Hutchinson Cancer Research Center is based partly on the previously mentioned study showing diminished relapse rates with an increased dose of TBI but at the cost of increased toxicity. It is hypothesized that by delivering radiation specifically to marrow, spleen, and sites of leukemia using an ^{131}I anti-CD45 monoclonal antibody, one should be able to increase the radiation dose specifically to sites of disease, thereby reducing relapse rates without increasing treatment-related toxicity *(36)*. In the current phase II study, patients with AML in first remission are being treated with a standard busulphan-cyclophosphamide preparative regimen combined with a dose of ^{131}I anti-CD45 sufficient to deliver roughly 2000 cGy to the marrow. When the first 27 patients entered in the study were analyzed, their disease-free survival at 4 yr was roughly 80%.

4.2. The Nonablative Approach

With many malignant diseases, most notably CML, it has become increasingly clear that the successful elimination of tumor following transplantation is, to a great extent, the result of an immunologic reaction of donor T-cells against host tumor cells. Evidence supporting this view includes the increased relapse rates seen following syngeneic as opposed to allogeneic transplantation, the increased relapse rates seen with aggressive T-cell depletion of donor marrow, the association of reduced relapse rates with the development of clinically obvious acute and chronic GvHD, and the ability of donor lymphocyte infusions to induce remissions in patients who have relapsed following transplantation. This evidence has led investigators to ask whether the graft-versus-leukemia effect of transplantation might be achieved without subjecting patients to the toxic effects of very high-dose therapy through the use of nonablative transplant regimens. In outbred canine models, Storb et al. *(37)* found that it is possible to achieve sustained complete engraftment with as little as 200 cGy TBI if posttransplant immunosuppression with cyclophosphamide and mycophenolate mofetil is given. By adding a small dose of fludarabine to the 200 cGy, it is possible to achieve complete sustained engraftment in humans even if they were heavily transfused.

The ability to achieve complete engraftment of donor marrow with little toxicity offers a number of possible therapeutic strategies that are now being explored. First, it is possible that the graft-versus-leukemia effect afforded by the transplant will be sufficient by itself to offer therapeutic benefit. Thus, nonablative transplants are now being explored as consolidation therapy for patients with AML who would not otherwise be candidates for a standard transplant by virtue of age or medical complications. Second, it now becomes possible to develop preparative regimens solely for their antileukemic effect without having to consider immunosuppression and to combine such therapies with a nonablative transplant approach. For example, studies are now under way combining targeted radiotherapy with the nonablative transplant preparation mentioned above. Finally, it may become possible to isolate donor T-cell clones with relative specificity for host leukemic cells and to use these after a nonablative transplant, as will be discussed next.

5. POSTTRANSPLANT THERAPIES

5.1. Donor Lymphocyte Infusions

Patients who relapse after allogeneic transplantation sometimes respond to the infusion of unirradiated donor lymphocytes. A summary of 258 patients reported by the European Registry noted complete responses in 75% of patients with CML, 38% with myelodysplasia, 24% with AML, and 15% with myeloma *(38)*. Responses were seldom seen in ALL. The major complication of donor lymphocyte infusions (DLIs) has been GvHD and myelosuppression, both of which can be severe or fatal. The risk of GvHD is influenced by both the dose and schedule of DLI, with an increased risk seen with a higher total cell dose and with bolus as opposed to fractionated dosing. Transfecting DLI with a suicide gene, such as the herpesvirus thymidine kinase, may provide a mechanism whereby lymphocytes can be infused and allowed to function as long as no adverse events occur, but then be destroyed in vivo by administering ganciclovir or a similar drug should GvHD develop. Investigations are also under way to explore whether use of a general subset of donor lymphocytes, such as just the CD4+ cells, improves the efficacy and safety of DLI, but as yet there are no comparative trials allowing one to make such a judgment.

In general, because of the potential toxicities of DLI, their use has been restricted to the treatment of patients who have relapsed after transplantation. More recently, some workers are asking whether a strategy of T-cell depletion of marrow with subsequent prophylactic DLI might have advantages over the more conventional transplant approach.

5.2. Minor Histocompatibility Antigens as Targets for T-Cell Therapy

To provide an antileukemic effect after allogeneic transplantation between matched siblings, donor lymphocytes must recognize either true tumor-specific antigens or, as seems more likely, minor histocompatibility antigens that differ between the donor and the recipient. Many of these minor antigens are expressed ubiquitously and therefore are targets not only for a graft-versus-leukemia effect but also for GvHD. Other antigens, however, are expressed by only a limited range of tissues, presumably reflecting tissue-specific functions of the proteins. Such antigens, if restricted to hematopoietic tissue, and if sufficiently polymorphic so as to differ between donor and recipient, might make ideal targets for posttransplant adoptive T-cell therapy or might be candidates for vaccine development. Recently, Warren et al. *(39)* have isolated CD8+ cytotoxic T-lympkocyte (CTL) clones specific for minor histocompatibility antigens from the peripheral blood from 10 allogeneic bone marrow transplant recipients. Among 19 clones analyzed, 14 recognized antigens expressed by hematopoietic cells of the patient and not by skin fibroblasts, suggesting that such cells might be able to induce a specific graft-versus-leukemia effect without inducing GvHD. The genes encoding several of these antigens have been identified, including a novel H-Y antigen and a minor histocompatibility antigen, denoted HB-1, selectively expressed by transformed B-cells. Similarly, Goulmy et al. *(40)* has characterized a number of CD8+ CTL clones designated HA-1–HA-7. Although many of these clones define antigens expressed by hematopoietic cells, endothelial cells, and fibroblasts, several, including HA-1 and HA-2, appear to be restricted in their expression to hematopoietic cells.

T-cell clones directed at minor histocompatibility antigens can lyse leukemic blasts in vitro and inhibit the outgrowth of leukemic colonies in soft agar. Furthermore, such T-cell clones can specifically prevent engraftment of human AML in the SCID mouse, demonstrating that the target antigens are expressed not only on the bulk of leukemic cells but also on the rare leukemia-initiating cell *(41)*. Clinical trials evaluating the use of T-cell clones specific for minor histocompatibility antigens are now being performed.

6. PROPHYLAXIS FOR GRAFT-VERSUS-HOST DISEASE

6.1. Pharmacologic Approaches

Although, as mentioned earlier, the risk of GvHD can be diminished with improved donor selection made possible with more sensitive tissue typing, even in the setting of HLA-matched siblings, and certainly with matched unrelated donors, acute and chronic GvHD continues to be a substantial problem. For more than two decades, the most frequently used form of GvHD prophylaxis has been a combination of cyclosporine plus a short course of methotrexate. Recently, two controlled randomized trials have been completed in which FK506 (tacrolimus) has been compared with cyclosporine, both being given with methotrexate *(42,43)*. In both studies, use of FK506 was associated with a lower incidence of acute GvHD, but in neither study was the frequency of chronic GvHD altered. Among matched siblings, overall survival was actually better in the cyclosporine arm, owing largely to a poorer outcome in patients with advanced disease treated with FK506. In the unrelated transplant setting, the use of FK506 was associated with a lower incidence of acute GvHD and less steroid use, but no difference in disease-free or overall survival. A randomized trial comparing methotrexate and cyclosporine with the same combination plus prednisone has also been completed *(44)*. No benefit was found for the three-drug combination over the two-drug regimen. Thus, based on results of randomized trials, there is little evidence that any pharmacologic approach is superior to the standard cyclosporine/methotrexate combination. In a canine model, the combination of mycophenolate mofetil plus cyclosporine seems to be very effective, and studies of this combination are under way in the clinic.

6.2. T-Cell Depletion

T-cell depletion of donor marrow can greatly reduce or eliminate both acute and chronic GvHD. However, in most previous trials of this approach for the treatment of acute leukemia, there was little evidence for improvement in disease-free survival or overall survival, owing to an increase in graft rejection (which was frequently fatal), opportunistic infections, and relapse. In an effort to overcome the problems of GvHD and relapse, investigators at Sloan-Kettering and elsewhere have experimented with intensified preparative regimens. In a pilot study of 31 patients with AML in first remission, the Sloan-Kettering group recently reported encouraging results, with approximately 75% of patients alive disease-free after receiving a regimen of cyclophosphamide, TBI, and thiotepa as

pretransplant conditioning, together with ATG and prednisone during the pretransplant period *(35)*. Other approaches to T-cell depletion that also look encouraging include the use of anti-T-cell antibodies with narrow specificity, add-back approaches in which after vigorous T-cell depletion, patients are given back a fixed low number of T-cells post transplant, and the use of low-dose posttransplant interleukin-2 (IL-2). To date, no published randomized trials of T-cell depletion have shown an overall advantage to this approach.

6.3. Biologic Agents for Prevention of GvHD

As our understanding of the basic biology of GvHD has advanced, it has become possible to develop specific biologic interventions, many of which are now in clinical trials. Inflammatory cytokines, such as IL-1 and tumor necrosis factor-α (TNF-α), which are released by damaged tissue following exposure to high-dose therapy, upregulate adhesion molecules and HLA antigens, which in turn may increase the risk of GvHD development. Once GvHD has developed, the same cytokines may directly cause further tissue damage. In animal models, the use of IL-1 receptor antagonists, soluble IL-1 receptor, antibodies to TNF, and soluble TNF receptor have all been shown to have activity in preventing GvHD and accordingly are now in clinical trials.

T-cell activation requires presentation of the appropriate antigen by an antigen-presenting cell in the presence of costimulatory molecules including B7 (CD80 and CD86). This complex interacts with the T-cell receptor and CD28 on the T-cell, resulting in T-cell activation. If the T-cell receptor is engaged without the B7-CD28 interaction taking place, tolerance results, at least in many animal models. Hence, strategies to block this interaction have been developed, including the use of anti-B7 antibodies, an antibody to CD28, or the use of CTLA-4Ig, a synthetic molecule that binds to B7 and blocks its interaction with CD28.

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34 Hematopoietic Stem Cell Transplantation

Perspective 2

VILMARIE RODRIGUEZ AND HELEN E. HESLOP

CONTENTS

1. INTRODUCTION

Many pediatric and some adult patients who present with acute leukemia can be cured with chemotherapy. For those with high-risk, relapsed, or refractory acute leukemia, however, hemopoietic stem cell transplantation (HSCT) provides better long-term disease-free survival (DFS) than does chemotherapy alone. Defining the appropriate candidates for HSCT is complicated by several variables including more precise definition of the risk factors that continually redefine the patient population. As many studies are retrospective, factors such as the intensity of previous chemotherapy and time of censoring often bias comparisons of transplantation with other treatment modalities. Many studies include both pediatric and adult patients, who have different prognoses. Finally, a much wider range of donors and stem cell sources is now potentially available to transplant specialists. In this chapter, we discuss indications for transplantation in various clinical settings, types of stem cell sources, and future directions.

2. INDICATIONS AND OUTCOME

2.1. Allogeneic Transplants from Matched Siblings

2.1.1. Acute Myeloid Leukemia

A number of registry and single-center studies show that the outcome of matched-sibling allogeneic SCT in patients with acute myeloid leukemia (AML) is worse when the disease has reached an advanced stage. In analyses of the International Bone Marrow Transplant Registry (IBMTR), there was an effect of age on survival following HLA-matched sibling transplants, with patients younger than 20 yr having superior survival to older patients *(1)*. Studies from Seattle have shown an association between cell dose and outcome, with patients who received more than the median cell dose having significantly less graft-versus-host-disease (GvHD) and regimen-related morbidity and improved DFS *(2)*.

Several studies have reported long-term cure with allogeneic HSCT in the range of 15–20% in AML patients who failed to achieve remission during induction *(3,4)*, and allogeneic HSCT is the treatment of choice in these patients or those with relapsed disease. The most contentious area is whether patients in first remission should receive an allogeneic transplant. Most recent single-center and registry studies show cure rates of 60–70% *(1,5)*, which is about 20–30% higher than the outcome with chemotherapy alone. IBMTR data show that the 3-yr probability of survival for HLA-identical sibling transplants for AML performed between 1991 and 1997 was 60% for patients transplanted in first complete remission *(1)*. Some single-center studies show higher DFS rates, with a study from Memorial Sloan-Kettering showing a 77% DFS rate for AML in first complete remission *(5)*.

A number of factors confound interpretation of the findings discussed above. All single-center or registry studies are subject to selection bias, so that a number of cooperative groups have performed randomized studies to compare outcomes in AML patients receiving chemotherapy alone or allogeneic or autologous BMT *(6–8)*. In most of these studies, patients with a matched sibling were randomized to allogeneic HSCT ("genetic" randomization), and analysis was based on intent to treat.

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Table 1
Disease-Free Survival in Randomized Studies Comparing Allogeneic Transplantation, Autologous Transplantation, and Chemotherapy in Patients with AML in First Remission

	Disease-free survival (%)		
	HSCT		
Study	*Allogeneic*	*Autologous*	*Chemotherapy*
MRC AML10 *(6)*	—	53	40
SWOG *(69)*	43	35	35
EORTC *(8)*	55	48	30
POG *(7)*	52	38	36
GOELAM *(70)*	50	44	40

Abbreviations: HSCT, hematopoietic stem cell transplantation; MRC, Medical Research Council; SWOG, Southwest Oncology Group; EORTC, European Organization for Research and Treatment of Cancer; POG, Pediatric Oncology Group; GOELAM, Groupe Ouest Est Leucemies Aigues Myeloblastiques.

Table 2
Disease-Free Survival of Patients Receiving Allogeneic Transplants for ALL in Second Remission

	Donor (%)	
	Matched sibling	*Unrelated*
IBMTR 1991–1997	42	33
POG/IBMTR *(13)*	40	
Memorial Sloan-Kettering *(71)*	62	
NMDP *(17)*		34
Bristol *(20)*		53

Abbreviations*: IBMTR, International Bone Marrow Transplant Registry; POG, Pediatric Oncology Group; NMDP, National Marrow Donor Program.*

The results, summarized in Table 1, vary widely. Unfortunately, in many of these studies randomization was incomplete, and many patients analyzed on an intent-to-treat basis in one arm actually received another therapy. In addition, by the time a study was mature and reported, new strategies potentially able to improve the efficacy of each treatment were under evaluation. Finally, although allogeneic HSCT convincingly reduces the risk of relapse, patients relapsing after chemotherapy have a higher likelihood of curative responses to salvage therapy; hence, the argument can be made that the highest overall cure rate is attained with a combination of chemotherapy and salvage HSCT for patients who relapse. At present, most investigators would recommend allogeneic transplantation for patients with high-risk features, such as chromosome 5 or 7 abnormalities, and chemotherapy for patients with good-risk features, such as t(8;21) or inv16. Selection of treatment for the intermediate group is more contentious and should probably be made on the basis of features of individual cases.

After relapse, allogeneic HSCT is the treatment of choice. IBMTR data show a DFS of 40% for patients in second complete remission, compared with 50% in a Memorial Sloan-Kettering study *(5)*. These results are similar regardless of whether bone marrow transplantation (BMT) was undertaken in early first relapse or second remission. Thus, most patients with a previously identified matched-sibling donor should proceed to allogeneic HSCT at relapse.

2.1.2. Acute Lymphoblastic Leukemias

In children with acute lymphoblastic leukemia (ALL), the likelihood of cure with modern intensive chemotherapy is 70–80% *(9)*. Allogeneic HSCT in first remission should be confined to patients whose leukemia has poor prognostic features, such as the t(9;22) or the t(4;11) translocation, or to patients with delayed responses to remission induction therapy. A recent multicenter study has confirmed that post treatment outcome in children with Philadelphia chromosome-positive (Ph+) ALL is superior with use of HSCT vs chemotherapy alone *(10)*. In adults, the cure rate with chemotherapy is only 30–40%, because more patients have high-risk features [age > 30 yr, white blood cells (WBCs) > 25×10^9/L, null cell phenotype, remission induction > 4 wk, extramedullary disease, or presence of t(9;22) or t(4;11)]. Any one of these features reduces the chance of long-term DFS with chemotherapy to 15–25%, therefore making the patient a candidate for allografting in first remission. IBMTR data show a DFS rate of 52% in adult patients receiving allografts in first remission *(1)*, whereas single-center rates range from 48 to 61% *(11,12)*. These results are difficult to interpret because of varying proportions of patients with different risk factors in each study. In a prospective French study, patients with matched sibling donors received allogeneic transplants, and others were randomized to autografting or chemotherapy *(11)*. The DFS rate for allograft recipients, 48%, did not differ significantly from that for autograft recipients or patients receiving chemotherapy (both 35%) *(11)*. However, among high-risk patients (Ph+, null or undifferentiated immunophenotype, age > 35 yr, or WBCs > 30×10^9/L), the DFS rate was significantly higher in the allograft vs the chemotherapy group (39% vs 14% at 5 yr) *(11)*.

After relapse, the chances of cure with chemotherapy are low in adults, and allogeneic HSCT should be performed. Results of this procedure undertaken in second remission are summarized in Table 2. IBMTR data from 1991 to 1997 show a DFS rate of 42% for patients transplanted in second remission *(1)*. For children who relapse on maintenance therapy, particularly after modern intensive chemotherapy, the chance of long-term survival with chemotherapy is <20%, mandating allogeneic HSCT. A combined Pediatric Oncology Group and IBMTR study yielded a DFS rate of 40% in patients receiving allografts, compared with 17% in those receiving chemotherapy *(13)*. The requirement for allografting in children with an isolated extramedullary relapse or who relapse more than 6 mo after completing maintenance therapy is more contentious, as these patients may be cured with salvage chemotherapy.

Once patients are beyond second remission, the results of all allografting procedures worsen considerably, with only 10–15% of patients becoming long-term disease-free survivors. A number of registry and single-center studies show that outcome is worse with an advanced disease stage *(14,15)*. Approximately 10–20% of patients with no response to primary induction therapy can be cured with allogeneic transplants from a matched sibling *(3)*.

2.2. Allogeneic Transplants from Alternative Donors

Fewer than a third of the patients who might benefit from allogeneic transplantation have an HLA-identical sibling; the remaining patients must consider use of an alternative donor. Historically, the mortality rate associated with this procedure has been higher than that for matched sibling HSCT *(16)*, owing to higher frequencies of graft rejection, GvHD and severe infections *(16)*. Data from the National Marrow Donor Program (NMDP) *(17)* on recipients with AML who received unrelated donor transplants in first complete remission show a 2–3-yr overall survival probability of 25%, whereas patients in second complete remission had a 3-yr probability of survival of 28%. For patients with ALL transplanted in first or second remission, the DFS rates were 34 and 32%, respectively. In contrast, the DFS rate was only 6% if HSCT was undertaken in relapse.

However, with the development of better molecular techniques for donor-recipient HLA matching and improvements in GvHD and infection prophylaxis, improved results after unrelated donor HSCT have been obtained. Several pediatric studies have reported outcomes similar to those obtained with matched-sibling transplantation *(18–20)*. Again, disease status was important. Whereas the DFS rate in the Seattle study was 47% for patients with acute leukemia transplanted in first or second remission, it was only 10% for patients transplanted in relapse or in greater-than-second remission *(19)*. Two studies showed outcomes not significantly different from the outcome with matched-sibling transplantation. The Milwaukee group reported a 60% DFS rate in patients with low-risk disease (acute leukemia in first or second remission or CML in chronic phase) and 34% for those with more advanced disease *(18)*. Hongeng et al. *(21)* reported DFS rates of 73 and 32% for children with standard-risk and high-risk disease, respectively. A Danish report on children undergoing HSCT for ALL actually shows a better outcome in children receiving matched-unrelated donor transplants: 3-yr DFS of 67% compared with 56% for those receiving grafts from family donors *(22)*.

2.3. Autologous Transplants

Autologous HSCT remains an investigational approach in the therapy for ALL. This procedure allows the use of higher doses of chemotherapy, but it carries the risk of reinfusion of leukemic cells in the harvested marrow. A recent comparison of autologous and unrelated donors performed at the University of Minnesota and the Dana-Farber Cancer Institute showed an increased risk of relapse after autograft and increased regimen-related toxicity after unrelated donor transplant *(23)*. DFS was better after transplantation from an unrelated donor. One potential use of autologous HSCT may be in patients who are poorly compliant with maintenance chemotherapy.

Autologous HSCT has been studied much more extensively in AML than in other leukemias *(24)*. Although gene marking studies have convincingly shown that infused leukemic cells can contribute to relapse after this procedure *(25,26)*, the role of purging has remained unclear. Several small studies suggest a benefit *(24)*, but there are no phase III studies to confirm this impression. Several large randomized trials have compared outcome after chemotherapy or autologous HSCT in patients with AML in first remission (Table 1). As discussed, all these studies have been hampered by low randomization rates. Although all show a reduced relapse rate after autologous transplantation, a significant benefit in DFS has been harder to demonstrate because of increased regimen-related toxicity. This problem may potentially be overcome by using mobilized peripheral blood rather than marrow, which should result in faster recovery of leukocyte counts. Different outcomes may also reflect variations in study design. For example, a better outcome was achieved when autologous HSCT was used in addition to rather than as a substitute for intensive chemotherapy *(6)*.

3. AREAS OF CURRENT RESEARCH INTEREST

3.1. Source of Stem Cells for Allogeneic Transplantation

3.1.1. Bone Marrow Versus Mobilized Peripheral Blood

Cytokine-mobilized allogeneic peripheral blood stem cell (PBSC) harvest has recently become an alternative to bone marrow as a source of stem cells for matched-sibling transplants. Early phase II studies showed that this source of stem cells resulted in faster engraftment and no increase in acute GvHD [perhaps due to a granulocyte colony-stimulating factor (G-CSF)-mediated shift to Th2 helper cells] but an increased incidence of chronic GvHD *(27,28)*. A recent prospective randomized study of allogeneic PBSCs compared with marrow showed a 2-yr actuarial overall survival of 45% in patients receiving marrow compared with 70% in those receiving PBSCs *(29)*. Differences in survival were significant for patients with unfavorable-risk diseases but not for those with favorable-risk diseases *(29)*. In a retrospective multivariate analysis from the IBMTR comparing the results of 288 HLA-identical sibling blood stem cell transplants with results of 536 HLA-identical sibling bone marrow transplants, the relapse incidence between the two transplant groups did not differ significantly *(30)*. However, treatment-related mortality rates were lower and leukemia-free survival rates were higher with use of blood stem cell transplants in patients with advanced vs early leukemia (acute leukemia in first remission or chronic myeloid leukemia in the chronic phase) *(30)*. Similarly, in a preliminary report of a randomized double-blind study in which 39 patients with malignant hematologic disorders were infused with either bone marrow ($n = 19$) or PBSCs ($n = 20$), a significantly lower risk of relapse was seen in the latter group *(31)*. The results of more studies should become available over the next few years, but the current experience suggests that peripheral blood should be the preferred source of stem cells for patients with high-risk disease. For patients with low-risk disease, the increased risk of chronic GvHD needs to be balanced against the risk of relapse.

3.1.2. Cord Blood

Another alternative source of stem cells that has been attracting much interest is cord blood. Several recent studies have demonstrated the feasibility of transplants with cord blood from unrelated donors *(32,33)*. Such transplants may have slower engraftment, but they also induce less GvHD. The immediate availability of cryopreserved cord blood units eliminates the usual delay in HSCT when unrelated donor marrow is used. There are concerns that the number of stem

cells present in cord blood may be insufficient to produce engraftment in adults, and for the moment most of these transplants are being performed in children or adolescents.

Locatelli et al. reported the outcome of 102 children with acute leukemia who received either related or unrelated umbilical cord blood HSCT *(34)*. In multivariate analysis, the most important factor influencing neutrophil engraftment was a nucleated cell dose $>3.7 \times 10^7$/kg. The most important factor influencing event-free survival (EFS) was disease status at the time of transplantation: good-risk patients had a 2-yr EFS rate of 49%, compared with 8% in patients with more advanced disease. Rubenstein et al. *(32)* reported the outcomes of 562 recipients of placental-blood transplant from unrelated donors. The speed of myeloid engraftment was associated with the leukocyte content of the graft, whereas transplantation-related events were associated with the patient's underlying disease and age, the number of leukocytes in the graft, the degree of HLA disparity, and the transplantation center.

3.1.3. Unrelated Donors

Historically, the outcome after transplantation from unrelated donors has been inferior to that observed after matched-sibling transplantation owing to an increased incidence of graft rejection and of GvHD resulting from increased alloreactivity in this setting *(16)*. Over the past few years, improved results have been reported from several single-center studies in defined patient populations, reflecting improvements in donor/recipient matching, GvHD prophylaxis, supportive care, and the timing of transplantation. In NMDP analyses, younger donor and recipient age were associated with significantly improved outcome *(14)*. As marrow donor registries have increased in size, the probability of finding a suitable donor has also increased. Indeed, 83% of all preliminary searches submitted to the NMDP in the United States identified at least one serologically identical HLA-A, -B, and -DR identically matched donor. As molecular matching for class I and perhaps minor antigen becomes more widely available, decisions will have to be made on which mismatches are best tolerated. In some settings, T-cells may recognize a single amino acid mismatch *(35)*. Conversely, detection of serologic or molecular differences that do not affect T-cell recognition can needlessly eliminate potential donors from consideration. The challenge will be to identify permissive mismatches appropriate for different types of GvHD prophylaxis regimens.

With the increasing success of matched-sibling peripheral blood transplantation, this stem cell source is being evaluated in the unrelated-donor setting. The likelihood of increased alloreactivity in this clinical setting makes the risk of acute GvHD a concern; hence, CD34 selection techniques initially pioneered in haploidentical transplants (*see* below) are being explored.

3.1.4. Haploidentical Family Donors

The genetic sharing of one chromosome of the chromosome 6 pair, containing the complete DNA code for the MHC, makes a haploidentical donor in essence a "half-matched donor *(36)*. There is also a greater likelihood for identity between MHC and minor histocompatibility antigens expressed from other chromosomes than could be expected between unrelated individuals. This opens another potential source of donors that increases the access to allogeneic HSCT. Use of a mismatched family member donor is associated with an increased risk of GvHD due to increased alloreactivity, and this risk increases with the degree of mismatch. Most studies therefore show that transplants from donors mismatched in a single antigen produce results equivalent to those achieved with matched-sibling donors, whereas outcomes are inferior with use of donors mismatched in two or three antigens.

Three main approaches have been explored to reduce the risk of GvHD. The first is T-cell depletion of the donor marrow in conjunction with immunosuppression both before and after transplantation. Henslee-Downey et al. *(35)* reported 2-yr overall survival rates of 55% in low-risk patients and 27% in high-risk patients who underwent haploidentical transplantation by this approach. A second strategy is to use G-CSF-mobilized, large-volume apheresis and CD34 selection with or without additional T-cell depletion. The reported DFS rates achieved with this alternative source of donors range from 17 to 40%. Aversa et al. reported that in a series of 43 patients with acute leukemia, the use of mobilized, large-volume apheresis and E-rosette T-cell depleted, CD34+ selected stem cells could achieve engraftment without GvHD *(37)*. Patients underwent a highly myeloablative and immunosuppressive conditioning regimen consisting of single-dose total-body irradiation (TBI), thiotepa, ATG, and fludarabine. DFS rates for AML and ALL patients were 36 and 17%, respectively. A different approach, was taken by the Dana-Farber group, which relied on the induction of anergy to inactivate alloreactive T-cells in the donor marrow *(38)*. In a preliminary report of 12 patients, bone marrow from a donor mismatched with the recipient for one HLA haplotype was cocultured with irradiated cells from the recipient for 36 h in the presence of CTLA-4-Ig, an agent that inhibits B7/CD28-mediated costimulation. Only 3 of 11 evaluble patients developed acute GvHD, and 5 patients were alive in remission *(38)*.

3.1.5 Autologous Marrow

Over the past few years clinical practice has changed so that autologous HSCT for diseases other than leukemia is almost always done using cytokine-mobilized peripheral blood rather than marrow. In patients with AML, there has been some concern about this strategy owing to the risk of mobilizing malignant cells. Indeed, a recent survey by the European Bone Marrow Transplant Registry showed that although blood cell count recovery was faster when peripheral blood was used, DFS was not significantly different compared with the result for unpurged marrow and was lower than in patients receiving purged marrow *(39)*. There is still interest in purging, and a recent review showed a significant advantage in DFS for recipients of purged marrow *(40)*. Another approach that might improve outcome is immune modulation post transplantation to stimulate the recovering immune system. Although studies with cytokines such as IL-2 have shown evidence of immuno-modulation, there are not as yet any phase III studies showing evidence of efficacy. Another approach that shows promise in preclinical and murine models is to vaccinate the patient with irradiated tumor cells transduced with cytokines such as granulocyte macrophage

(GM)-CSF *(41)* or immunostimulatory molecules such as CD40 *(42)* during the period of immune reconstitution.

4. DISEASE ERADICATION

Relapse is the major cause of failure after transplantation for acute leukemia. Strategies to reduce the risk of relapse include intensifying the conditioning regimen, altering the timing of transplantation, and augmenting the graft-versus-leukemia effect. A recent study showed that administering lower doses of cyclosporine could reduce the relapse risk and improve DFS in children undergoing HSCT for leukemia *(43)*.

4.1. Ablative Preparative Regimens

Conditioning regimens used for allogeneic HSCT must achieve adequate immunosuppression of the recipient to prevent rejection of the donor marrow cells and destroy residual malignant cells while causing minimal toxicity. Most preparative regimens for ALL use TBI and cyclophosphamide, a combination that produced a better outcome than busulphan-cyclophosphamide in a recent IBMTR study: 3-yr probabilities of survival and overall survival were 55% vs 40% and 50 vs 35%, respectively *(44)*. Because most chemoradiation regimens are at the limits of toxicity, any escalation to attempt reduction of the risk of relapse would probably increase regimen-related toxicity to unacceptable levels, particularly in older or heavily pretreated patients. Addition of biologic agents, such as monoclonal antibodies reactive with ALL cells or radioconjugates, may provide antileukemic activity without increasing toxicity. CD45 is one of the most broadly expressed antigens in malignant cells (90% of AML and most ALL cases), with a higher copy number per cell (200,000 vs 10,000 copies per cell for CD33) *(45)*. The Fred Hutchinson group tested the use of ^{131}I-labeled anti-CD45 with a preparative regimen of busulphan-cyclophosphamide. Among 44 patients in this phase I study, 37 showed a favorable distribution of the isotope. Of 25 evaluable patients, 10 survived post-transplantation for a median of 58 mo, with 11 relapses and 4 deaths from infection *(45)*.

4.2. Subablative Regimens

High-dose chemotherapy and allogeneic stem cell transplantation carry a substantial treatment-related morbidity and mortality in older patients (older than 50 yr), those with compromised organ function (e.g., congestive heart failure), or coexisting infections or in those who were heavily pretreated prior to HSCT. In all these patients, treatment-related mortality can exceed 50%, making them ineligible for HSCT. More recently, new strategies for allografting have explored an approach of less intensive conditioning therapy given with the sole aim of facilitating allogeneic engraftment. This strategy is based on the success of donor lymphocyte infusion (DLI) in eradicating malignancy and preclinical studies in a canine model, thus demonstrating the feasibility of reducing conditioning therapy without comprising engraftment or antileukemic activity. A variety of regimens based on low-dose TBI or fludarabine are under investigation *(46–49)*. The major problem with submyeloablative regimens is an increased rate of graft failure, ranging from 5 to 30% vs 1 to 5% in patients who underwent full myeloablation prior to HSCT. The use of lymphodepleting antibodies or a combination of monoclonal antibodies in addition to cytotoxic or immunosuppressive drugs could potentially decrease rejection rates *(50)*.

5. POSTTRANSPLANT THERAPIES

5.1. Donor Lymphocyte Infusions

Adoptive immunotherapy with DLI provides a means of augmenting the graft-versus-leukemia effect after allogeneic HSCT to eliminate residual disease. Approximately 70% of all relapsed chronic myeloid leukemia patients treated in the chronic phase achieve complete cytogenetic remission with this approach *(51)*. For other patients with hematologic malignancies, relapsing after transplant therapy with DLI has resulted in a much lower response rate: 29% of patients with AML and 5% with ALL *(52)*. There is some evidence that use of immunostimulatory cytokines such as interleukin-2 may amplify graft-versus-leukemia mechanisms and induce remissions in patients who have failed to respond to DLIs *(53)*.

DLI is associated with toxicity and treatment-related complications. The incidence of GvHD varies from 55 to 90% *(51)*. Pancytopenia and bone marrow aplasia are most likely to occur in patients with advanced disease, including accelerated phase or blast crisis. Treatment-related mortality rates have been reported to be approximately 20% (13% bone marrow aplasia or infection and 7% complications associated with GvHD) *(51)*. One means of reducing the risk of GvHD is to administer antigen-specific cytotoxic T-cell lines (CTL) lines when a specific antigen is known. Potential targets include minor antigens differentially expressed on hemopoietic cells *(54)* or lineage-specific antigens, such as WTI or proteinase 3 *(55)*. Such lines could potentially mediate cytotoxic activity directed at recipient hemopoiesis (and leukemia) but not donor hemopoiesis.

5.2. Prophylaxis and Therapy of Infectious Complications

Reconstitution of the recipient immune system is delayed after transplantation and may be further delayed by immunosuppressive therapy for GvHD prophylaxis or treatment *(56)*. Improvements in prophylaxis and therapy of viral and fungal infections may contribute to an improved outcome. Cytomegalovirus (CMV) infection has been a significant cause of posttransplant mortality, and seronegative patients have had better outcomes *(57)*. The advent of effective pharmacologic prophylaxis with ganciclovir and intravenous immunoglobulin has improved the prognosis for CMV-seropositive patients. However, a recent study showed that although preemptive ganciclovir prevents CMV disease, CMV seropositivity remains an independent adverse risk factor *(58)*. Thus, alternative treatments for prophylaxis against CMV have been explored. The Seattle group infused clones of CD8+ CMV-specific CTLs into 14 patients 30–40 d after HSCT *(59)*. Cytotoxic activity against CMV was increased post infusions in 11 patients who were deficient in such activity, but responses were maintained only in patients who had recovery of specific CD4-mediated responses against CMV. A follow-up study in which both CD4 and CD8 clones were transferred has shown long-term persistence of transferred immunity *(60)*. Patients trans-

planted with T-cell-depleted marrow from matched unrelated or mismatched family member donors have had a high incidence of Epstein-Barr virus (EBV)-induced immunoblastic lymphoma, a complication linked to the immunodeficiency that follows this procedure *(61–63)*. Therapy with unmanipulated T-cells or antigen-specific CTLs has proved effective against this complication *(62,64–66)*. Furthermore, in a prophylaxis study, administration of EBV-specific CTLs to >50 recipients of T-cell-depleted grafts from unrelated donors reduced the incidence of EBV lymphoproliferation from 11.5 to 0% *(66)*.

5.3. GvHD PROPHYLAXIS

GvHD results from alloreactivity between donor and recipient. The two major prophylactic regimens employed to prevent this complication are pharmacologic (administration of immunosuppressive drugs) and immunologic (in vitro T-cell depletion of the donor marrow). The standard pharmacologic prophylaxis has been cyclosporine and short-course methotrexate, but recent studies suggest that the incidence of GvHD is even lower if FK506 is substituted for cyclosporine *(67)*. MMF also shows promise in animal models, and its combination with cyclosporine is being evaluated in clinical trials. Ex vivo T-cell depletion reduces the risk of both acute and chronic GvHD and may allow higher tolerance of mismatching but may also increase the risk of rejection and delay immune reconstitution. A confounding feature for interpreting the value of T-cell depletion is that a variety of methodologies are employed to remove T-cells, including physical methods and monoclonal antibodies. Some techniques produce a pan-T-cell depletion, whereas others use antibodies with more restricted T-subset specificities. A recent IBMTR study shows a better outcome when antibodies with narrow specificities are used *(68)*. Nevertheless, a large NMDP study comparing pharmacologic immunosuppression with T-cell depletion did not show a significant difference in outcome, although the range of posttransplant complications differed by mode of prophylaxis *(14)*.

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V BIOLOGIC TREATMENTS

A *HEMATOPOIETIC GROWTH FACTORS*

35 Hematopoietic Growth Factors in Patients with Acute Leukemia

CHARLES A. SCHIFFER

CONTENTS

1. INTRODUCTION

The myeloid colony-stimulating factors [granulocyte (G)-CSF and granulocyte/macrophage (GM)-CSF] were first approved for use as a means of attenuating the duration of neutropenia following chemotherapy administration in patients with solid tumors. This probably still represents the most common indication for which these drugs are prescribed. Recognizing the potential for overuse of these expensive drugs, a number of national organizations including the American Society of Clinical Oncology (ASCO) created guidelines for the utilization of these products in different clinical circumstances *(1)*. The original ASCO guidelines, published in 1994, have recently been updated with the benefit of further clinical trials performed for many different diseases *(2)*. The data available at the time the initial guidelines were written were relatively sparse, and the large number of gray areas allowed considerable clinician discretion and may have actually contributed to increased use of growth factors. Also contributing to the widespread use was a quirk in some insurance reimbursement policies in the United States such that insurance coverage for certain parenteral medications is only provided when the medications are administered in a physician's office. Because there is remuneration to the physician for these activities, this created an inherent conflict of interest, which could have influenced prescribing patterns for growth factors at either a conscious or subconscious level.

As detailed in the updated guidelines, the recent randomized trials evaluating growth factors as primary prophylaxis against infection following standard chemotherapeutic regimens have generally either been considered "negative" by their authors or "positive" because of statistically significant shortening of neutropenia, but with marginal effects on other clinical end points. There has also been widespread use of growth factors as a means of treating febrile episodes occurring in neutropenic patients, with little evidence to support this approach *(3)*.

2. GROWTH FACTOR USE IN PATIENTS WITH ACUTE LEUKEMIA

The use of G-CSF and GM-CSF has been evaluated extensively in multiple large clinical trials in patients with acute myeloid leukemia (AML) during the last 10–15 yr, with the goal of reducing infectious morbidity and mortality and thereby potentially increasing the response rate *(4–14)*. Indeed, the number of trials of similar design that were done is striking, given the remarkable consistency of the findings of these studies. In brief, it is generally acknowledged that the growth factors shorten the duration of neutropenia following induction chemotherapy by a few days compared with control groups receiving either placebo (in the more rigorous trials) or no cytokine, with no effect on remission rate, duration of response, incidence of severe infections, or survival (Table 1) *(15)*. Although some studies showed modest reductions in days of hospitalization and antibiotic use, these were inconsistent findings, and criteria for hospital discharge and antibiotic use, including the concurrent use of prophylactic coverage with quinolone antibiotics, were rarely stipulated explicitly in the publications. When considered in aggregate,

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Table 1
Effects of Myeloid Growth Factors During Therapy of Acute Myeloid Leukemia

- Consistent, modest reduction in the duration of neutropenia (multiple randomized trials)
- More pronounced shortening of neutropenia following postremission consolidation therapy
- No effect on CR rate, CR duration or survival
- Variable effect on duration of hospitalization and antibiotic use

Abbreviations: CR, complete remission.

Table 2
Myeloid CSFs: Possible Explanations for Limited Benefits

- Cannot affect overall outcome of leukemia unless death or morbidity from infection or inability to deliver "full-dose" chemotherapy is limiting
- Do not eliminate neutrophil count nadirs with a narrow time window to detect benefit
- Recovery time to 100–200 neutrophils/μL may not differ appreciably between growth factor-treated and -untreated patients
- Unknown effect of endogenous levels of cytokines during severe cytopenia

these trials did ameliorate earlier concerns about the potentially harmful effect of stimulation of cell growth in patients with AML, although this can still probably occur in individual patients. Thus, growth factors should be stopped in patients who have rising blast counts following induction therapy. Although more studies were done in patients with AML, similar conclusions were reached in the induction therapy trials conducted in both adults and children with acute lymphoblastic leukemia (ALL) *(16,17)*.

It should be noted, however, that there is consistent evidence from two randomized studies *(10,18)* and one historically controlled evaluation *(19)* of what appears to be a clinically important reduction in both the duration and perhaps the depth of neutropenia in AML patients treated with growth factors following postremission consolidation therapy. Although survival and remission duration were not affected in these trials, there is considerable potential for reduction or elimination of the need for hospitalization, and adjunctive therapy with G- or GM-CSF can be recommended in this circumstance.

Perhaps the most exciting and innovative trials evaluated pretreatment with cytokines to induce cycling of leukemic progenitors, with the aim of enhancing the cytotoxic effects of therapy. Unfortunately, randomized trials utilizing GM-CSF as priming prior to and/or during the administration of chemotherapy to patients with AML have not resulted in improved outcome *(12,14,20)*, despite promising in vitro data supporting such an approach *(21,22)*. This brief overview focuses on possible explanations for why these biologically very potent agents were not more effective and also comments on future directions for the development of therapeutic cytokines.

3. FACTORS LIMITING THE EFFECTIVENESS OF GROWTH FACTOR THERAPY IN PATIENTS WITH LEUKEMIA

There are a number of reasons why the results of growth factor therapy in leukemia patients have been less exciting than initially hoped (Table 2). Supportive care modalities such as growth factors cannot have an impact on tumor response and overall patient outcome unless infection-related treatment mortality and morbidity are significant causes of treatment failure or an inability to delivery "full" doses of therapy. Although infectious deaths are an important cause of treatment failure, particularly in older adults, antibiotic therapy has improved considerably, and the most serious infectious problems are usually secondary fungal infections, occurring most frequently in patients presenting with bacterial infections at the time of diagnosis or in those who require a second course of induction therapy. (Parenthetically, the safest way to schedule growth factor administration for patients requiring additional courses of induction chemotherapy is still unclear).

Because the depth of neutropenia is not affected, and most patients with leukemia present with neutropenia, there is only a relatively brief window in which the beneficial effect of the growth factor might become apparent. Thus, patients receiving induction therapy for AML do not receive the growth factor during the first week of chemotherapy, when most are already neutropenic. They then have relatively aplastic marrows for the next week to 10 d with continued severe neutropenia and counts first begin to recover 3–4 wk after therapy is completed. Thus, a 1–3 d decrease in days with neutrophil counts $<500/\mu L$ at the end of induction would be predicted to have little effect on the frequency of infections and modest impact on the duration of hospitalization and antibiotic use, since most infections develop in the earlier part of treatment. Indeed, further analysis *(23)* of the only trial purporting to show a reduction in the incidence of infections *(5)* suggests that the difference was limited to the small subgroup of patients who received a second course of induction therapy. Because further increases in the dose intensity of antileukemic therapy are precluded by toxicity to nonhematopoietic organs, the growth factors have not allowed delivery of much higher doses of induction therapy. Furthermore, with the possible exception of a timed sequential chemotherapeutic approach in children with AML *(24)*, it has not been shown that delivery of arbitrarily defined increases in dose intensity is feasible or of benefit to adults with leukemia.

The end point of neutrophil count recovery in the randomized trials can also be questioned. As noted above, most studies reported that the time to recovery to 500 or 1000 neutrophils/μL is accelerated by growth factor use. However, much lower neutrophil levels are well tolerated for long periods by patients with chronic, primary marrow disorders. Antibiotic trials in neutropenic patients have repeatedly shown that elevations in peripheral neutrophil count of $\leq 100/\mu L$ result in stabilization or rapid recovery from infection *(25)*, presumably because the blood neutrophil count underestimates the actual mass of neutrophils available to fight or prevent infection. Indeed, it is well established that neutrophils can be found in inflammatory sites well in advance of obvious rises in the

peripheral blood neutrophil count *(26)*. Thus, although growth factors do accelerate rises in counts to levels of 500 or 1000/μL, this may not be the most important goal, and the available studies have not provided information about any differences in time to recover to 100–200 neutrophils/μL.

There have been no systematic studies of the levels of endogenous myeloid CSFs during periods of neutropenia. It has been shown, however, that endogenous thrombopoietin levels are markedly increased during periods of therapy-induced thrombocytopenia *(27)*. It is therefore possible that marrow precursors are already maximally stimulated and that pharmacologic doses of exogenous growth factors might have modest additive effects. In fact, a series of small randomized trials in patients with AML evaluating thrombopoietin have consistently failed to demonstrate accelerated platelet count recovery or decreased requirements for platelet transfusions, presumably for many of the same reasons that limited the benefits from the myeloid CSFs *(28–30)*.

A variety of ancillary research end points have been sidebars in many of the growth factor studies including cost-benefit calculations as well as assessments of impact on quality of life. Although both end points are obviously relevant in some clinical circumstances, such analyses are also often done when more obvious clinical benefits are not obvious. Moreover, some of these "studies" may be driven as much by marketing as by "scientific" considerations. For example, if there is no impact on response or survival with a possible shortening of hospitalization of a few days, it is relatively simple to calculate the cost of additional hospitalization vs the number of days of growth factor administration and to determine inferentially both a competitive price for the growth factor and a marketing strategy.

4. OTHER APPROACHES AND FUTURE DIRECTIONS

The myeloid growth factors only affect neutrophil recovery. An ideal "protective" agent would abolish clinically important cytopenias during repetitive cycles of therapy, have no stimulatory effect on tumor growth, eliminate the serious and dose-limiting problem of gastrointestinal tract mucositis, and, if one were really grandiose, would also keep the urine flowing, the liver metabolizing, and the heart ticking without cumulative damage to these and other organs. On the assumption that there are common inflammatory mediators of some of these chemotherapy side effects, studies with such expanded goals have begun with inhibitors of compounds such as tumor necrosis factor (TNF). Although it is probably unlikely that a single agent could provide such widespread "protection," trials with a variety of different classes of compounds are either in progress or in the planning stages.

Recent trials with lisofylline (LSF) in AML and in patients after marrow transplantation have been disappointing. LSF is an inhibitor of phosphatidic acid generation that has been shown to modify inflammatory injuries to the gastrointestinal tract and lung in some preclinical models, possibly by inhibition of elaboration of TNF-α and other inflammatory cytokines *(31)*. The results of a multicenter randomized trial evaluating the potential protective effect of 3 mg/kg of LSF given every 6 h for 28 d added to standard induction therapy for newly diagnosed patients with AML were recently presented *(32)*. One hundred sixty-five patients were randomized in this double-blind trial. There was no difference in the number of serious infections in the two groups, which was the primary study end point; nor was there any difference in overall outcome from treatment. Multiple other outcomes were assessed, some of which marginally favored the LSF group, including the complete remission rate in a subgroup of patients aged 45–59 yr. It is unclear, however, why this particular age group was plucked out, given the wide age range (23–82 yr) of patients enrolled in the study.

These results are consistent with the outcome of a smaller randomized trial of 70 patients conducted at M.D. Anderson Cancer Center that showed no differences in rates of infection, mortality, time to count recovery, and postremission survival in patients receiving LSF or placebo *(33)*. A small study that enrolled 20 patients/arm compared two doses of LSF vs placebo in recipients of HLA-matched allogeneic bone marrow transplant from HLA-identical siblings *(34)*. There was no difference in the time to hematologic recovery among the groups, although there was a decrease in the rate of documented infections favoring the LSF group, as well as improved 100-d survival. Given the size of the study as well as the heterogeneity of the recipients, it is unclear whether these are benefits that can be extrapolated to a larger population. In addition, current practices have switched to the use of peripheral blood as the source of stem cells, with much shorter periods of marrow aplasia. There is also great interest in the use of nonablative stem cell transplant approaches that substantially decrease acute regimen-associated toxicities. Thus, even if this study were to be considered unequivocally "positive," its relevance to future practice would be limited. Lastly, all studies noted increased nausea and vomiting in LSF recipients.

If LSF is not the answer, the question remains. Many modulators of cytokine-mediated injury are being studied in a variety of oncologic *(35)*, rheumatologic, and gastrointestinal chronic inflammatory disorders, including thalidomide and some of its analogs, [which potentially have fewer undesirable side effects *(36)*] as well as etanercept (Enbrel®), a TNF-α inhibitor recently licensed for the treatment of refractory rheumatoid arthritis *(37)* and keratinocyte growth factor and Interleukin-11, with their potential protective effects on gastrointestinal mucosa *(38,39)*. These may eventually merit evaluation as a means of reducing chemotherapy-associated toxicity. Amifostine, which has received U.S. Food and Drug Administration approval for amelioration of cisplatin-induced renal dysfunction and radiation-induced mucositis in patients receiving therapy for upper airway malignancies, also has possibilities as a "protect it all" drug. The appreciable infusion-related side effects from amifostine (which requires daily administration) could conceivably complicate already complex acute leukemia therapy. A phase I trial combining amifostine with standard-dose cytarabine and escalating doses of idarubicin is in progress; whether dose escalation of idarubicin can be achieved and whether this would have any impact on overall outcome will not be known for quite some time *(40)*.

What is the next decade and next generation of agents likely to bring? There are growth factors, such as c-kit ligand, that

have more "proximal" actions on less differentiated stem cells *(41)*. Other "chimeric" compounds, which are combinations of different cytokines, are being evaluated with the hope of synergistic effects on blood count stimulation and stem cell mobilization *(42)*. However, it is probable that there is only modest room for further decreases in the duration of cytopenia given what appears to be a requisite "lag" period required for full cellular maturation following stimulation of normal hematopoietic precursors.

More hypothetically, one wonders whether this suppression of normal hematopoiesis seen in patients with acute leukemia, particularly AML, might be instructive. Individuals with AML acquire a mutation in an early hematopoietic precursor with the development of a clone that has a proliferative advantage over normal cells. When the clone reaches a certain size, normal hematopoiesis is profoundly suppressed, with the resultant cytopenias associated with this disease. The mechanism(s) by which this inhibition of hematopoiesis occurs is poorly understood, but it also seems to confer significant protection against the effect of cytotoxic therapy, in that if the antileukemic effects are sufficient, there is rapid and predictable proliferation and repopulation by normal hematopoietic elements. Thus, whatever produces this suppression may also be protective against the effects of chemotherapy; if characterized further, it could prove to be of appreciable clinical interest. Whether this observation is simply a consequence of a cytokinetic change in the normal stem cell population with an increase in the number of cells in G0, or whether other mechanisms of drug resistance are present, merits further study. Future considerations could include the use of a "protector" compound, followed by administration of CSFs to enhance the rate of recovery.

5. CONCLUSIONS

The first decade of hematopoietic growth factors usage has produced benefits for large numbers of patients and major increases in our understanding of the biology of hematopoiesis. In the clinical arena, there has probably been overuse of these agents, with appreciably less impact on overall treatment outcome than had been hoped. Compounds are under evaluation with the potential to serve more as protective than as rescue agents, and we look forward to the results of these ongoing and planned studies.

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36 Hematopoietic Growth Factor Treatment in Leukemia

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CONTENTS

1. INTRODUCTION

The process of hematopoiesis is regulated by a large number of glycoprotein cytokines that orchestrate the growth and development of hematopoietic cells, from the pluripotent self-renewing stem cell through committed lymphoid and myeloid progenitor and precursor cells to nonreplicative, functionally mature blood cells. The control of hematopoiesis and the migration and functional activation of blood cells are closely controlled by the stimulatory and inhibitory effects of at least 25 regulatory factors known as hematopoietic growth factors or cytokines *(1,2)*.

Some cytokines have a broad spectrum of activities (e.g., interleukin-3), some mainly influence the survival and/or development of primitive, non-lineage-restricted multipotent progenitor cells (e.g., stem cell factor), and others principally promote the growth of cells with a more restricted developmental potential [e.g., granulocyte and granulocyte-macrophage colony-stimulating factors (G-CSF and GM-CSF)]. G-CSF is lineage-specific for granulocyte precursors, whereas GM-CSF is less restricted and influences the proliferation and differentiation of erythroid, megakaryocytic, and myeloid lineage progenitor cells *(1–3)*.

Several hematopoietic growth factors have been isolated, biochemically characterized, and produced by recombinant DNA techniques in quantities sufficient for clinical use *(4–6)*. Recombinant forms of G-CSF and GM-CSF are used in various clinical applications *(7,8)*. The most commonly available recombinant G-CSFs are filgrastim and lenograstim. Filgrastim is expressed in *Escherichia coli* and differs from the naturally occurring molecule by the addition of an N-terminal methionine and the absence of glycosylation *(4)*. Lenograstim is expressed in mammalian (Chinese hamster ovary) cells and is glycosylated *(5)*. GM-CSF is available in Europe as molgramostim, a nonglycosylated product expressed in *E. coli*, and in the United States as sargramostim, a yeast-derived glycosylated protein that differs from the native molecule by a single amino acid substitution *(6)*. The physiologic and clinical relevance of different patterns of glycosylation and other minor differences between the recombinant proteins and their endogenous counterparts is unknown.

Administration of recombinant G-CSF causes a sustained and dose-dependent increase in circulating neutrophils that is maintained during daily administration. There is a shortening of time required for neutrophil precursors to mature and appear in the circulation, from approx 5 d to 1 d *(7)*. GM-CSF is a multilineage factor that mainly induces proliferation of granulocyte, macrophage, and mixed granulocyte-macrophage colonies of bone marrow cells *(3)*. It increases neutrophil numbers in humans, but the increase is less marked and slower than that observed with G-CSF *(8)*.

It also increases neutrophil motility at low concentrations and inhibits neutrophil migration at high concentrations, possibly to immobilize these cells at sites of infection. Neutrophil respiratory burst, adherence, phagocytosis, and bacterial killing are enhanced in vivo after therapy with either G-CSF or GM-CSF *(9)*. Neutrophils induced by G-CSF and GM-CSF treatment (in patients not suffering from primary defects in neutrophil function) show grossly normal functional abilities *(9)*.

The broader activity of GM-CSF appears to confer an immunomodulatory role in enhancing antigen processing and presentation by monocytes. Administration of GM-CSF to patients with malignancies results in induction of MHC class II

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expression on monocytes, which is necessary for presentation of antigen to T-helper lymphocytes *(10)*. GM-CSF is involved in the development of dendritic cells from progenitor cells and from peripheral blood monocytes, although CD40 ligand rather than GM-CSF appears to be the most important factor in this process *(11)*. Both G-CSF and GM-CSF mobilize transplantable hematopoietic progenitor cells from the bone marrow to the blood stream, an effect that was not expected from in vitro studies. Long-term reconstituting cells are present in the circulation but only at a low concentrations. Levels increase in response to chemotherapy or G-CSF or GM-CSF therapy. Animal studies show that after transplantation, long-term regeneration occurs through proliferation and differentiation of donor stem cells rather than recovery of host stem cells *(12)*. Recombinant G-CSF is now used routinely to mobilize peripheral blood progenitor cells (PBPCs) and to augment the mobilizing effects of chemotherapy. Although it is not known with certainty whether stem cells with significant long-term reconstituting potential are present in the mobilized PBPC product, the numbers of hematopoietic progenitor cells expressing the membrane antigen CD34 correlate well with early neutrophil and platelet engraftment and with the incidence of colony-forming unit (CFU)-GM in PBPC populations. The mechanism for progenitor cell mobilization by G-CSF and GM-CSF is not established, but it may involve interference with the activity of extracellular adhesion molecules and/or their receptors involved in the adhesion of CD34+ cells to marrow stromal elements.

2. CLINICAL APPLICATIONS OF G-CSF AND GM-CSF

2.1. Chemotherapy Support

Myelosuppression is one of the most common dose-limiting toxicities of cytotoxic drugs used at standard curative doses in the treatment of malignancies. Although the resulting neutropenia may not in itself be life-threatening, the risk of infection is closely related to the depth and duration of neutropenia *(13,14)*. An important consequence of neutropenia is the need to reduce chemotherapy doses or to delay treatment until blood counts have recovered, leading to a decrease in dose intensity and a potential adverse effect on survival *(15)*.

The use of G-CSF to reduce chemotherapy-induced toxicity can be divided into primary prophylaxis (given before the occurrence of neutropenia) and secondary prophylaxis (given in subsequent chemotherapy cycles after the occurrence of prolonged neutropenia or febrile neutropenia in an earlier cycle). They may also be used in the treatment of established febrile neutropenia, although efficacy appears to be less marked than with prophylactic use. The American Society of Clinical Oncology has published recommendations for the use of hematopoietic growth factors (ASCO guidelines) *(16)*.

2.1.1. Prophylactic Treatment

Given as primary prophylaxis, treatment with G-CSF after cycles of chemotherapy shortens the incidence and duration of severe neutropenia, with improvements in associated clinical end points, such as the incidence of infection, duration of hospitalization, and requirement for intravenous antibiotics in patients with solid tumors and hematologic malignancies *(17–20)*. It allows chemotherapy to be delivered at close to the planned dose intensity *(18,19)*. Two prospective, randomized, placebo-controlled trials have shown efficacy for initiation of G-CSF therapy with the first cycle of chemotherapy (i.e., primary therapy) in reducing the incidence of febrile neutropenia by approximately 50% in patients receiving cytotoxic chemotherapy *(17,18)*.

The Crawford et al. study *(17)* was a multicenter, prospective, randomized, placebo-controlled trial involving 211 patients with small cell lung cancer (SCLC). Placebo or G-CSF (230 $\mu g/m^2$/day subcutaneously) was administered after as many as six cycles of CAE (cyclophosphamide, doxorubicin, etoposide) chemotherapy. The duration and severity of neutropenia were significantly reduced in patients receiving the growth factor. During cycle 1, the rate of febrile neutropenia was reduced by 50% and the difference in the cumulative event rate across all cycles was statistically significant. Antibiotic use and number of days of hospitalization were also significantly reduced by about 50% with filgrastim. Owing to the crossover design of the trial, many patients randomized to the placebo group were eventually allowed to receive open-label filgrastim, which makes interpretation of the results more complicated.

A phase III, multicenter, prospective, randomized, placebo-controlled trial confirmed the above results, showing in addition the improved ability to deliver chemotherapy as scheduled in patients receiving G-CSF *(18)*. In this study, 130 patients with SCLC also received G-CSF (230 $\mu g/m^2$/d) or placebo after CAE chemotherapy. Over all cycles, the growth factor significantly reduced the incidence of febrile neutropenia and there was a significant reduction in the requirement for parenteral antibiotics, and a significant reduction in the number of days of hospitalization. G-CSF significantly reduced the duration and severity of neutropenia and the need to delay or decrease the dose of chemotherapy.

In two prospective randomized studies in children with acute lymphoblastic leukemia (ALL), G-CSF reduced the incidence of febrile neutropenia and improved adherence to the tight time frame of the curative chemotherapy protocol *(21,22)*. The incidence of febrile neutropenia was reduced in the study by Welte et al. *(21)* from 40 to 17% ($p < 0.007$), the number of documented infections from 15 to 8% ($p = 0.04$), and the time requiring intravenous antibiotics from 32.2 to 18.2 d ($p = 0.02$). In the study by Pui et al. *(22)*, the documented infections were reduced from 36 to 16% ($p = 0.009$) and days in hospital from 10 to 6 ($p = 0.11$). Similar results have also been obtained by other investigators *(23–25)*. The dependence of the clinical benefit from hematopoietic growth factor treatment on the intensity of the chemotherapeutic protocol used is well illustrated by the study of Laver et al. *(26)* in children with T-cell leukemia. This study showed that G-CSF has no effect after induction chemotherapy with a short period of neutropenia, whereas during maintenance therapy, in which the median time of neutropenia was longer (11 d), the growth factor was capable of reducing the incidence of febrile neutropenia ($p = 0.017$). However, one study of G-CSF in children with ALL did not demonstrate any effects on end points such as

febrile neutropenia, infections, or days in the hospital *(27)*. Thus, it can be postulated that the effects of G-CSF become more significant when myelosuppression is prolonged *(25,26)* or when chemotherapy regimens include repeated cycles *(21)*.

G-CSF and glycosylated GM-CSF (sargramostim) are also used after chemotherapy in patients with acute myeloid leukemia (AML). In a large double-blind, placebo-controlled study of AML patients aged 55 yr or older, G-CSF accelerated neutrophil recovery following induction chemotherapy, and patients had significantly fewer days with fever or antibiotic treatment *(28)*. In another large study *(20)*, it significantly reduced the duration of neutropenia and improved infection-related end points after induction and consolidation chemotherapy, as well as reducing the duration of hospitalization. In older patients, sargramostim produced a faster neutrophil recovery after induction chemotherapy, with reduced incidences of infections and fatal pneumonia, fever, and induction therapy-related deaths *(29)*. Two studies of the use of molgramostim (non-glycosylated GM-CSF) in elderly AML patients after induction chemotherapy showed faster neutrophil recovery but no evidence of clinical benefit *(30,31)*. Another study found no effect on neutropenia and a prolonged duration of thrombocytopenia *(32)*. Although leukemic blasts, especially myeloid leukemic blasts, express receptors for G-CSF and GM-CSF, concerns that the use of growth factors will have an adverse effect on leukemic cell proliferation owing to stimulation of cytokine receptors on these cell appear generally unfounded *(31,32)*, and there does not appear to be any adverse effect on response rates *(20)*. In one study in children with ALL, GM-CSF did not have any effect on febrile neutropenia, infections, or hospitalization *(33)* but significantly reduced the requirement for intravenous antibiotics.

Thus far, studies have not been specifically conducted with the aim of demonstrating that the use of G-CSF to support delivery of planned dose intensity translates into a long-term survival advantage. However, none of the randomized studies performed (see above) demonstrated any change in the event-free survival (Table 1).

2.1.2. Interventional Treatment

In the daily clinical situation, G-CSF is more often administered interventionally compared with prophylactic treatment. However, either G-CSF or GM-CSF compete with potent combinations of antibiotics. Only in situations in which the severe neutropenias last for >1 wk, does G-CSF or GM-CSF in combination with antibiotics have a potential advantage over antibiotic treatment alone. Indeed, in randomized studies with G-CSF *(34)* or GM-CSF *(35)* in patients with leukemias (and solid tumors) harboring febrile neutropenias, the interventional administration of these growth factors significantly reduced the days in hospital and the time requiring intravenous antibiotics. However, none of the studies testing interventional administration of G-CSF or GM-CSF demonstrated a significant reduction of documented infections or survival advantage *(34,35)*.

There are but few published data on the administration of G-CSF or GM-CSF in patients with documented severe infections or sepsis. A nonrandomized study of G-CSF in patients with leukemia failed to demonstrate a clinical benefit *(36)*. In a study in neutropenic patients with deep-seated fungal infections, G-CSF plus amphotericin B was significantly superior in reduction of the infection, compared with amphotericin B alone ($p = 0.027$) *(37)*.

2.1.3. Conclusions

1. In patients receiving *intensive* or repeated cycles of chemotherapy, such as those with high-risk ALL, G-CSF reduces the incidence and duration of febrile neutropenia, the number of documented infections, and the duration of time in the hospital.
2. In patients with febrile neutropenia, either G-CSF or GM-CSF marginally reduced the requirement for intravenous antibiotics or hospitalization.
3. There are no data showing that G-CSF has any effect on survival, either by reducing the number of deaths caused by severe infections or by increasing the response to chemotherapy (e.g., by increasing the dose of chemotherapy over time).
4. There is no cost benefit from using hematopoietic growth factors.

2.1.4. Recommendations

G-CSF or GM-CSF should be used only as prophylactic therapy in high-risk leukemia patients receiving high-dose intensive chemotherapy or repeated cycles of intensive chemotherapy.

2.2. Bone Marrow or Peripheral Blood Progenitor Cell Transplantation

The myeloablative, high-dose chemotherapy used in conjunction with bone marrow transplantation (BMT) is followed inevitably by a period of bone marrow aplasia. The administration of G-CSF in the post-BMT period has been shown to accelerate neutrophil recovery and to improve associated clinical end points *(38,39)*. A reduction in post-BMT morbidity has also been reported with the use of GM-CSF *(40,41)*. In comparative terms, G-CSF may produce more rapid neutrophil engraftment post BMT *(42,43)*, with associated improvements in fever and hospitalization *(44)*. GM-CSF (sargramostim) is also approved in the setting of graft failure or delay, defined in terms of low absolute neutrophil counts (ANCs), with or without infection.

Sheridan et al. *(45)* was the first to report the superiority of G-CSF in terms of accelerated hematopoietic recovery when filgrastim-mobilized PBPCs were reinfused with autologous bone marrow after high-dose chemotherapy, compared with the use of bone marrow alone. Schmitz et al. *(46)* reported a randomized comparison of filgrastim-mobilized PBPCs and autologous BMT in lymphoma patients treated with high-dose chemotherapy, showing significant reductions in time to platelet recovery, requirement for platelet transfusions, and neutrophil recovery, with fewer red blood cell transfusions required and less time spent in the hospital. A subsequent economic analysis of this study showed a significant cost saving *(47)*, mostly due to the lower costs of autograft collection, shorter hospital stay, and reduced need for supportive care in patients receiving PBPCs.

Table 1
Selected Randomized Studies with G-CSF and GM-CSF in Patients with Acute Leukemia

			Improvement of quality of life			*Influence on ASCO end points*[a]			
Cytokine (dose)	*Disease*	*No.*	*Reduction of febrile neutropenia*	*Reduction of documented infections*	*Reduction of intravenous antibiotics*	*Reduction of hospital stay*	*Improvement of survival*	*Cost savings*	*Reference*
G-CSF									
10 μg/kg/d, sc	ALL	32	NS	NS	—	NS	—	—	Dibenedetto et al., 1995 *(27)*
G-CSF (filgrastim)									
5 μg/kg/d, sc	ALL (high-risk)	34	$p = 0.007$	$p = 0.04$	$p = 0.02$	—	NS	—	Welte et al., 1996 *(21)*
10 μg/kg/d, sc	ALL	164	NS	$p = 0.009$	NS	$p = 0.011$	NS	NS	Pui et al., 1997 *(22)*
5 μg/kg/d, sc	AML	521	$p = 0.009$ or NS	NS	$p = 0.0001$ or NS	$p = 0.001$	NS	—	Heil et al., 1997 *(22)*
10 μg/kg/d, sc	T-cell leukemia and lymphoma (stages III–IV)	88	—	—	—	NS	NS	NS	Laver et al., 1998 *(26)*
200 $\mu g/m^2$/d, sc	ALL, AML	17	$p = 0.0023$	NS	$p = 0.0006$	$p = 0.0001$	—	—	Chen et al., 1998 *(23)*
5 μg/kg/d, sc	ALL,T-NHL	17	NS	NS	NS	$p = 0.01$	—	—	Clarke et al., 1999 *(24)*
G-CSF (lenograstim)									
5 μg/kg/d, sc	ALL (high-risk)	67	$p = 0.005$ or NS[b]	—	$p = 0.005$ or NS[b]	$p = 0.005$ or NS[b]	NS	—	Michel et al., 2000 *(25)*
GM-CSF (regramostim)									
5.5 μ/kg/d, sc	ALL	40	NS	NS	NS	NS	NS	—	Calderwood et al., 1994 *(32)*
GM-CSF (molgramostim)									
5 μ/kg/d, sc	ALL	119	NS	NS	$p < 0.001$		NS	S	Saarinen-Pihkala et al., 2000 *(33)*

Abbreviations: CSF, colony-stimulating factor; G, granulocyte; GM, granulocyte/macrophage; NS, not significant; S, significant; T-NHL, T-cell non-Hodgkin's lymphoma.
[a] See ref. *16* for American Society of Clinical Oncology (ASCO) guidelines.
[b] Dependent on chemotherapy regimen.

Comparative studies in healthy volunteers have shown G-CSF to be more effective than GM-CSF in terms of the numbers of CD34+ cells mobilized *(48,49)*. The administration of G-CSF at a dose of ≥ 10 μg/kg/d subcutaneously for 5–6 consecutive d, followed by two to four leukapheresis procedures, will allow the collection of ≥ 2×10^6 CD34+ cells/kg body weight in the vast majority of patients. A study comparing the two CSFs in a mobilization regimen in conjunction with chemotherapy found no differences in CD34+ cell yield or in engraftment *(50)*. In a study comparing G-CSF and GM-CSF with high-dose cyclophosphamide for PBPC mobilization in multiple myeloma patients, both mobilization regimens were effective *(51)*. Although GM-CSF is an effective mobilizing agent, moderate bone pain and low-grade fever mean that some patients are less likely to tolerate it in this setting.

Studies have also explored the potential synergy between CSFs. In one study, the addition of G-CSF to GM-CSF increased mobilization (of colony-forming units, GM and burst-forming units, erythrocytes) compared with either agent alone and with the reverse sequence *(52)*. A combination of low-dose cyclophosphamide followed by sequential GM-CSF (5 d) and G-CSF (5 d) was more efficient in mobilizing CD34+ cells than G-CSF alone *(53)*. Spitzer et al. *(54)* found no difference in mobilization (mononuclear cells, CD34+ cells) and questionable clinical benefit with G-CSF + GM-CSF vs G-CSF alone. Clinical trials comparing the mobilization efficacy of the two available forms of recombinant G-CSF (filgrastim and lenograstim) showed no significant differences between the two forms *(55,56)*. G-CSF may also be used for mobilizing PBPCs in healthy donors participating in allogeneic transplantation *(57)*. In a multicenter European Group for Blood and Marrow Transplantation study of allogeneic transplantation, filgrastim-mobilized PBPCs provided comparable engraftment to BMT, with earlier platelet recovery, whereas filgrastim administration and leukapheresis in normal donors were both feasible and well tolerated *(58)*.

The benefit of hematopoietic growth factors after allogeneic bone marrow transplantation in children with leukemias is documented in several studies *(59–62)* in which G-CSF and GM-CSF significantly reduced the severity and duration of febrile neutropenias. In one study, G-CSF reduced the median hospital stay from 37 to 28 d ($p < 0.01$) and the requirement for intravenous antibiotics from 19 to 7 d ($p < 0.001$) *(60)*. There was no documented influence on the development and severity of graft-versus-host disease. None of the studies demonstrated an influence of G-CSF or GM-CSF on survival. The feasibility of using PBPCs has also been reported in children with hematologic malignancies *(63)*.

2.2.1. Conclusions

1. G-CSF or GM-CSF are potent mobilizers of hematopoietic progenitor and stem cells from the bone marrow into the peripheral blood.
2. The G-CSF- or GM-CSF-mobilized stem and progenitor cells are capable of reconstituting hematopoiesis after myeloablative chemotherapy and/or irradiation.
3. G-CSF- and GM-CSF hasten the recovery of myelopoiesis after autologous or allogeneic bone marrow or PBPC transplantation, leading to a reduction in both febrile neutropenias and hospitalization.

2.2.2. Recommendation

Both G-CSF and GM-CSF are recommended for stem cell mobilization and for ameloriation of neutropenia post transplantation.

2.3. Severe Chronic Neutropenia

The safety of long-term daily administration of G-CSF is convincingly documented in patients with severe chronic neutropenia (SCN), a term describing a group of rare conditions, including congenital neutropenia, cyclic neutropenia, and idiopathic neutropenia, all characterized by an ANC of $< 0.5 \times 10^9$/L. Patients suffer repeated episodes of fever, oropharyngeal inflammation, gastrointestinal symptoms, perirectal inflammation, cutaneous infections, and severe bacterial infections. Deep tissue infections and bacteraemias may also occur. Since other host defense mechanisms remain intact in the absence of immunosuppressive drugs or prolonged antibiotics, chronic viral infections or infections with intracellular pathogens are rare.

Before the use of G-CSF as a treatment for SCN, there was no reliably effective treatment for this condition. G-CSF dramatically improves the quality of life in these patients. Phase III studies showed restoration of ANC levels to $< 1.5 \times 10^9$/L in most patients, along with a significant improvement in clinical end points (incidence of infection, fever, oropharyngeal ulcers, and antibiotic use), with the daily filgrastim dose titrated to response *(64)*. Interestingly, GM-CSF treatment had no effect on neutrophil production in this patient group *(65)*. Following approval of filgrastim for long-term therapy of SCN, the Severe Chronic Neutropenia International Registry was established to monitor the clinical course, treatment, and complications of patients with SCN, including those receiving filgrastim treatment. Registry data *(66)* on >700 patients show that the dose and schedule of filgrastim required to restore a normal ANC are variable, and the dose required is generally lower in idiopathic or cyclic neutropenia (median doses of 1.0 and 1.7 μg/kg/d). These doses can be used as starting doses and titrated up or down at 1- or 2-wk intervals according to response. With prolonged use, the benefits of filgrastim use appear to be sustained. The development of antibodies to filgrastim has not been observed.

Almost all patients respond to G-CSF with increased neutrophils, reduced infections, and improved survival. Some responders with congenital neutropenia have developed myelodysplastic syndrome followed by acute myeloid leukemia (MDS/AML), raising the question of the role of G-CSF in pathogenesis. The Severe Chronic Neutropenia International Registry has data on 731 neutropenic patients, including 387 with congenital neutropenia, who were treated with G-CSF from 1987 to the present. The 387 patients with congenital disease were observed for a mean of 6 yr, (range, 0.1–11 yr) while being treated. Of these patients, 35 developed MDS/AML, for a crude rate of malignant transformation of nearly 9% *(67)*, contrasted with none of the 344 patients with idiopathic or cyclic neutropenia. Transformation was associated with acquired marrow cytogenetic clonal changes: 18 patients developed a partial or complete loss of chromosome 7, and 9

manifested abnormalities of chromosome 21 (usually trisomy 21). No significant relationships between age at onset of MDS/AML and patient gender, G-CSF dose, or treatment duration were found ($p > 0.15$). Although our data do not support a cause-and-effect relationship between the development of MDS/AML and G-CSF therapy or other patient demographics, we cannot exclude a direct contribution of G-CSF in the pathogenesis of MDS/AML. This issue is unclear because MDS/AML was not seen in cases of cyclic or idiopathic neutropenia. Improved survival of congenital neutropenia patients receiving G-CSF therapy may allow time for the expression of the leukemic predisposition that characterizes the natural history of these disorders. However, other factors related to G-CSF therapy may also be operative in the setting of congenital neutropenia.

Interestingly, patients with severe congenital neutropenia associated with progression toward MDS and AML have demonstrated G-CSF receptor mutations *(68,69)*. The G-CSF receptor mutations in patients with AML/MDS were present in cells of the myeloid lineage only and were nonsense mutations leading to truncation of the C-terminal cytoplasmic region crucial for maturation signaling. In 11 of 12 severe congenital neutropenia patients who developed AML, both the mutated and the normal alleles of the G-CSF receptor were expressed *(69)*. These findings support the notion that mutations in the G-CSF receptor gene, resulting in the truncation of the C-terminal maturation domain, are associated with progression from SCN to MDS/AML. The evolution from G-CSF receptor mutation to overt AML has occurred over several months and years, suggesting a considerable variation in these patterns *(66)*. From the data reported thus far we conclude that the point mutations in the G-CSF receptor gene occur spontaneously and are not inherited *(69)*. It is important to emphasize that patients with either cyclic or idiopathic neutropenia are not at risk for the development of AML/MDS *(67)*.

2.4. Granulocyte Transfusions

The use of granulocyte transfusions as an approach to the prevention and treatment of infection in neutropenic patients has attracted renewed interest in the availability of CSFs as a means to mobilize and collect adequate numbers of neutrophils. Use of filgrastim, with or without a corticosteroid, to improve preapheresis neutrophil levels has been found to provide high numbers of neutrophils with normal functional properties for transfusion therapy. Significant and sustained increases in ANCs were observed when granulocytes were transfused to HLA-matched allogeneic BMT recipients *(70–72)*. The need for supplemental platelet support can arise as a result of platelet contamination of the apheresis product. Further studies of granulocyte transfusions in allogeneic and autologous transplant patients are under way.

2.5. Safety

G-CSF is well tolerated by most patients: Bone pain appears to be the most frequent adverse effect of therapy and is generally controlled with mild analgesics *(7)*. The broader functional activity of GM-CSF increases its potential to cause side effects through the release of proinflammatory cytokines *(73)*. Although GM-CSF causes a number of adverse effects, including lethargy, myalgia, bone pain, anorexia, weight change, skin eruptions, and flushing, these are generally tolerable. Erythematous lesions may occur at the site of injection *(74)*.

3. SUMMARY AND CONCLUSIONS

The clinical development of hematopoietic growth factors in the early 1990s altered the practice of oncology by permitting cytotoxic chemotherapy to be administered at doses and schedules that were previously limited by severe neutropenia and associated complications. The initial anticipation that wider use of hematopoietic growth factors might lead to an improvement of survival was not fulfilled. However the quality of life of subgroups of patients was improved, as judged by fewer days of fever or hospitalization. Also, the hypothesis that hematopoietic growth factors can push leukemic cells into the cell cycle by interacting with their receptors, subsequently rendering them more sensitive for cycle-specific chemotherapy, did not translate into better long-term survival rates. Wider use of hematopoietic growth factors for the mobilization of PBPCs in transplantation was an equally important therapeutic advance, as it provided an alternative to bone marrow as the source of stem cells for haematologic support, conferring clinical benefits and savings in resources and costs. G-CSF has also provided an effective treatment for the group of diseases termed severe chronic neutropenia, for which no effective therapy was previously available. Their efficacy in surmounting neutropenia and mobilizing progenitor cells has also been exploited in different disease areas, for example, HIV-associated neutropenia and PBPC transplantation for autoimmune diseases, and the immunomodulatory effects of GM-CSF continue to be elucidated for potential applications in disparate fields, such as cancer vaccination and boosting influenza vaccinations. The colony-stimulating factors have also facilitated research into new areas, such as ex vivo expansion of hematopoietic cells, in which retroviral gene transfer studies will continue to advance our understanding of the processes of hematopoiesis and engraftment. The clinical roles for these useful agents will expand with the development of new formulations and the addition of recombinant forms of more hematopoietic growth factors and cytokines, providing further tools with which to manipulate the complex process of human blood cell development.

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V Biologic Treatments

B *Immunotherapy*

37 Immunotherapy of Leukemia

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CONTENTS

1. INTRODUCTION

The prospect of utilizing immunotherapy to improve the cure rate of childhood cancer is highly attractive, given the potential specificity of this approach and the potential absence of overlapping toxicity with current conventional therapies. One of the first observations that immunologic mechanisms may play a role in curing leukemia was made by Barnes and colleagues in 1956 *(1)*. They noted that in a murine bone marrow transplant (BMT) model, allogeneic recognition of leukemic cells by the graft might be required to cure the animals. The investigators also alluded to one potential drawback of this approach, namely, alloreactivity against normal host tissues, now known as graft-versus-host disease (GvHD). The existence of a graft-versus-leukemia (GvL) effect in human stem cell transplantation was first described by Weiden and the Seattle transplant team *(2)*, who noted a substantially lower risk of leukemic relapse among patients who developed GvHD, compared with those who did not. This observation was subsequently confirmed by retrospectively analyzing large cohorts of BMT patients.

Horowitz et al. *(3)* reported data from the International Bone Marrow Transplant Registry demonstrating that the risk of leukemic relapse was significantly higher among recipients of identical twin (syngeneic) grafts, compared with that for recipients of HLA-matched allogeneic grafts. A higher relapse rate was also seen in patients who failed to develop clinically significant GvHD and in those given T-cell-depleted grafts, suggesting that allorecognition by T-cells plays an important role in mediating the antileukemic effect. Interestingly, although the probability of relapse inversely correlated with the extent of GvHD, survival probability did not. Patients who developed severe (grade III or IV) GvHD had a significantly greater risk of transplant-related mortality and lower survival than did patients without GvHD. However, the development of mild GvHD was associated with a superior survival rate, indicating that "a little" GvHD is advantageous but that severe GvHD significantly compromises outcome. This observation underscores the delicate balance encountered when attempting to potentiate the immune response against leukemic cells, without enhancing reactivity toward normal tissues. Although the well-documented GvL effect requires the presence of an adoptive allogeneic hematopoietic system, many patients with potentially fatal hematopoietic malignancies cannot receive ablative therapy and a stem cell transplant. For them, any attempts to induce an immune-mediated antileukemic effect cannot rely on the function of an allogeneic marrow.

In this chapter we present approaches to immunotherapy of leukemia from two perspectives: the patient with a functioning hematopoietic allograft and the patient without such a graft. We discuss what is known about the immunobiology of the GvL effect and outline various clinical strategies to improve the antileukemic efficacy of allogeneic cell therapy and to reduce the risk of associated complications. Then we discuss ways in which the immune system may be manipulated to destroy leukemic cells in the absence of a functioning stem cell allograft. These include (1) the administration of cytokines following chemotherapy or autologous stem cell transplantation; (2) the utilization of monoclonal antibodies or their derivatives to purge autologous stem cell grafts or to facilitate eradication of leukemia cells in vivo; and (3) vaccination approaches to augment T-cell responses against leukemia-associated antigens.

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2. IMMUNOTHERAPY FOR PATIENTS WITH A STEM CELL ALLOGRAFT

2.1. The GvL Effect

2.1.1. Initiators of GvL

The GvL reaction is initiated when donor lymphocytes recognize foreign antigens present on normal or leukemic cells in the recipient, usually in conjunction with class II or I MHC antigens. This leads to clonal expansion of the alloreactive lymphocytes and generation of stimulatory cytokines, which recruit effector cells capable of mediating cytotoxicity against leukemic cells. At least four types of lymphocytes may be involved in mediating a GvL effect. CD4+ T-cells, which recognize class II MHC antigens, CD8+ T-cells, which recognize class I MHC antigens, CD56+ [natural killer (NK)] cells, and γδ T-cells. The latter two cell types can elicit MHC unrestricted killing. In addition, other cells capable of eliciting an immune/inflammatory response (monocytes and neutrophils) and B-cells may be involved in the GvH reaction and thus may also play a role in the antileukemic GvL phenomenon.

There is considerable evidence suggesting that CD4 cells play an important role in mediating the GvL effect. In a murine transplant model, depleting the marrow graft of CD8+ cells resulted in a low risk of GvHD but excellent leukemia-free survival *(4)*. In humans, CD4+ lymphocytes isolated from patients after allogeneic BMT or from control donors were capable of lysing cryopreserved allogeneic leukemia cells *(5,6)*. Jiang et al. *(7)* were also able to isolate cytotoxic T-lymphocytes (CTLs) that showed reactivity against the patients' leukemia from allogeneic transplant recipients *(7)*. Depletion of CD4+ cells from this effector population significantly reduced the precursor frequency of leukemia-reactive cytotoxic lymphocytes. Additional data supporting a central role for CD4+ cells in mediating GvL comes from studies evaluating the efficacy of T-cell subset infusions. Transplants utilizing CD8-depleted marrow grafts reduced the risk of GvHD without a concomitant increase in leukemic relapse *(8)*. Moreover, Giralt et al. *(9)* reported that chronic myeloid leukemia (CML) patients who relapsed after allogeneic BMT frequently achieved a second remission after donor lymphocyte infusions (DLIs) that had been depleted of CD8+ lymphocytes. Similarly, Alyea et al. *(10)* treated relapsed CML patients with CD8-depleted DLIs, using escalating doses of CD4+ cells. Complete cytogenetic remissions were achieved in 87% of patients with early-phase CML. Excellent responses were also observed in patients with relapsed multiple myeloma.

Although substantial data show that CD4+ cells are crucial in initiating the GvL reaction, it is likely that other lymphocyte subsets are also involved. In several murine transplant models, the use of CD8-depleted grafts resulted in higher rates of leukemic relapse *(11,12)*. Moreover, Palathumpatet et al. *(13)* demonstrated that supplementing a murine marrow graft with highly purified CD8+ cells augmented the GvL effect *(13)*. In humans, alloreactive CD8+ lymphocytes generated in vitro have demonstrated antileukemic activity *(14)*. There have been several reports of transplant protocols that included CD4-depleted grafts to reduce the risk of GvHD *(15–18)*. In most instances, a fixed or escalating number of CD8 cells was added to minimize the risk of graft rejection. Although data from the Seattle group suggested a possible increase in leukemic relapse for patients receiving CD4-depleted grafts, other investigators did not observe an increased relapse risk, suggesting a role for CD8+ lymphocytes in eliciting an antileukemic response *(15,16)*.

Other lymphocytes that may be involved in generating GvL activity include NK cells and γδ T-cells. Hauch et al. *(19)* reported that the inability to generate interleukin-2 (IL-2)-activated NK cell cytotoxicity against autologous CML targets in allogeneic transplant recipients correlated with an increased risk of posttransplant relapse *(19)*. Similarly, Jiang et al. *(20)* noted that the persistence of low NK cell numbers after allogeneic BMT for myeloid leukemia also correlated with an increased probability of relapse. These data suggest a role for NK cells in mediating immune surveillance after allogeneic stem cell transplantation.

γδ T-cells represent a subset of CD3+ lymphocytes that are able to lyse either virally infected cells or allogeneic tumors in an MHC-unrestricted manner. This unique population of cells may also be involved in mediating a GvL effect. γδ T-cell clones that exhibit strong cytotoxicity against autologous tumor cells have been isolated from patients with acute lymphoblastic leukemia (ALL) *(21)*. Malkovska et al. *(22)* reported that infusions of γδ T-cells into SCID mice (previously injected with lethal doses of Daudi lymphoma cells) significantly prolonged survival of the animals. No human trials utilizing infusions of γδ T-cells have been reported, and the extent and nature of their involvement in the GvL reaction remain uncertain.

Hence, multiple cell populations may be involved in initiating a GvL effect. It is possible that the type(s) of cells mediating GvL will vary depending on histocompatibility differences between donor and recipient, antigenic determinants present on the malignancy, and factors affecting the host's ability to mount an immune response, such as exposure to immunosuppressive therapy.

2.1.2. Targets for GvL

In order for antigens expressed on leukemic cells to be recognized by donor T-cells and initiate a cytotoxic response, several criteria must be met. The leukemia cell must be able to process and present antigen in association with appropriate class I or II MHC antigens, to express a costimulatory signal (e.g., B7) required for clonal expansion, and possibly to bear adhesion molecules (e.g., leukocyte function-associated antigen-3) to facilitate cellular interactions. The absence of one or more of these components may allow tumor cells to escape immunologic destruction. Antigens that might be targeted by the GvL response can be divided into three broad categories: (1) leukemia-specific antigens; (2) minor histocompatibility antigens (mHAs); and (3) tissue-restricted antigens (e.g., differentiation antigens) expressed on hematopoietic cells.

It is attractive to postulate that neoantigens expressed on leukemic cells, possibly resulting from chromosomal translocations or point mutations directly involved in leukemogenesis, could be recognized and destroyed by donor lymphocytes. Examples of candidate antigens include the promyelocytic leukemia-retinoic acid receptor α (PML-RARα) and BCR-ABL fusion proteins seen in acute promyelocytic leukemia (APL) and CML, respec-

tively *(23,24)*. Many other chromosomal translocations have been described in acute leukemia, such as those generating the *TEL-AML1* and *MLL-AF4* fusion genes, which could potentially result in new or aberrant antigen expression, thereby eliciting an immune response. Thus, although many hematologic malignancies possess genetic alterations (point mutations, deletions, or translocations) that contribute to the neoplastic process, it remains uncertain whether these "tumor-specific molecules" will be able to function as efficient "tumor-specific antigens" and whether patients will be able to mount a clinically meaningful response against them. For example, investigators have demonstrated that T-cell immunity can be generated against autologous cells expressing peptides from the BCR-ABL fusion protein found in CML, a disease that is particularly susceptible to allogeneic cell immunotherapy *(25)*.

However, it has been difficult to document strong responses against autologous leukemia cells expressing the BCR-ABL peptide. Whether this oncoprotein functions as a major antigen in triggering a GvL response following stem transplantation or DLI remains to be determined. Similarly, Gambacorti-Passerini et al. *(23)* have shown that T-cells can be generated that recognize peptides from the RARα fusion protein seen in APL. It was possible to generate such responses with T-cells from healthy control donors by putting the RARα peptide into autologous target cells from the donors. In contrast, when they evaluated this same type of immune reaction in patients with APL, it was very difficult to document specific reactions to RARα *(23)*, despite normal immune responses by these patients to other antigens recognizable by their T-cells. The conclusion suggested by these findings is that T-cells of the APL patients have somehow been "tolerized" by prolonged in vivo exposure to APL cells, making them incapable of mounting a response to the APL-associated RARα peptide.

The close association between GvL and GvH in HLA-identical sibling transplants suggests that minor histocompatibility antigens play an important role in the immunologic recognition of leukemic cells following allogeneic transplantation. These antigens may be ubiquitously expressed on host tissues or restricted to certain histologic lineages. For example, HA-3 and H-Y are expressed on skin-derived fibroblasts, keratinocytes, renal epithelial cells, and hematopoietic tissue, whereas HA-1 and HA-2 expression is restricted to cells of the lymphoid or myeloid lineage *(26)*. Ubiquitously expressed antigens would be expected to elicit a GvH as well as a GvL effect, although it is possible that leukemic targets might be more susceptible to cytotoxic pathways than are normal host cells *(14)*. Minor histocompatibility antigens restricted to hematopoietic tissue should elicit a more specific GvL response, since normal host hematopoietic cells would have been destroyed by the pretransplant conditioning regimen. It would therefore be attractive to utilize CTLs that recognize lineage-restricted mHAs for adoptive immunotherapy. Mutis et al. *(27)* were able to generate CTLs against HA-1 and HA-2 from healthy donors using dendritic cells pulsed with synthetic peptides. These clones could be expanded ex vivo and were capable of lysing allogeneic acute myeloid leukemia (AML) and ALL targets.

Finally, differentiation antigens such as CD19 (B-cells) or CD5 (T-cells) could potentially serve as targets for CTLs, if they were aberrantly expressed on leukemic cells. Allelic differences in these antigens could also result in recognition by donor T-cells, thereby initiating an immune response.

2.1.3. Effector Mechanisms

Once antigenic determinants on a leukemic clone are recognized by the donor's immune system, tumor cell eradication can occur through a variety of mechanisms. T-lymphocytes (CD4 and CD8) are capable of directly mediating cytotoxicity through a perforin/granzyme-B pathway *(28)*. Perforin alters cell membrane permeability, allowing granzyme-B to enter the cell and initiate apoptosis. Another mechanism by which T-cells induce tumoricidal activity is through the fas/fas-ligand pathway. Activated T-cells show surface expression of fas-ligand, which can bind to fas molecules expressed on target cells, triggering a chain of intracellular events leading to apoptosis. CML cells have a greater density of fas molecules on their surface than do normal hematopoietic stem cells and for this reason might be especially vulnerable to T-cells *(29,30)*. T-cells can also indirectly facilitate cytotoxicity by secreting cytokines that recruit and activate other effectors capable of mediating leukemic cell death. CD4 lymphocytes are predominantly responsible for this phenomenon and are classified into two subsets (Th1 and Th2) based on their cytokine secretion profile. Th1 cells are cytotoxic and produce inhibitory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) that can directly impede tumor cell growth. Th1 cells also produce IL-2 and IL-12, which activate NK cells and augment their cytolytic function. IL-2 also activates T-cells in an autocrine loop, causing expansion of the alloreactive clone. Th2 cells function in a helper-regulatory capacity and secrete cytokines such as IL-3, IL-4, and granulocyte/macrophage colony stimulating factor (GM-CSF). GM-CSF activates macrophages and monocytes, thus facilitating antigen presentation and enhancing the ability of these cells to participate in antibody-dependent cellular cytotoxicity.

2.2. Manipulation of the GvL Effect

Since recognizing the importance of GvL in eradicating human hematologic malignancies, research physicians have sought to capitalize on this effect as a form of biologic therapy. Several different posttransplant strategies have been explored, including (1) modification of GvHD prophylaxis; (2) use of allogeneic lymphocyte infusions; (3) administration of immunostimulatory cytokines; and (4) induction of autologous GvHD. In addition, there has been a trend toward treating patients with nonmyeloablative or "mini"-transplant regimens in efforts to reduce toxicity, while relying more heavily on the GvL effect to promote cure. Considerable effort has also been focused on devising methodologies that may dissociate GvL from GvHD in order to minimize damage to normal host tissues.

2.2.1. Modification of GvHD Prophylaxis

Cyclosporine A (CSA) is the most commonly used posttransplant immunosuppressive agent in the prevention of GvHD. It blocks the second stage of T-cell activation by inhibiting IL-2 synthesis *(31)*. Since the inhibition of T-cell activation induced by CSA is nonspecific, dose reduction or withdrawal of CSA might lead to enhancement of the GvL

effect. Indeed, rapid discontinuation of CSA in CML patients, relapsing after allogeneic transplantation, was occasionally sufficient to induce molecular remissions *(32)*. Complete responses have also been observed in some patients with AML and ALL *(33)*. Withdrawal of CSA has been associated with an increase in CTLs, as well as NK cells, both of which may be helping to mediate the antileukemic effect *(34)*. Unfortunately, not all the observed responses were durable, and some patients died of overwhelming GvHD. Nonetheless, this relatively simple therapeutic manipulation has allowed some patients to achieve prolonged leukemia-free survival and possibly cure, despite their relapse after intensive chemo-radiotherapy.

2.2.2. Donor Lymphocyte Infusions

The use of allogeneic lymphocyte infusions from a marrow transplant donor was first reported by the Seattle team, as a way to overcome graft rejection for patients with aplastic anemia *(35)*. This practice was subsequently abandoned because of an increased risk of chronic GvHD in a patient population in which the development of GvHD did not confer any survival advantage. Although it could not have been appreciated at the time, DLI was to become a promising therapy for leukemia patients relapsing after allogeneic transplantation. The therapeutic options for such individuals have generally been quite limited, and second marrow transplants were previously considered the only potentially curative approach. However, exposing patients to a second myeloablative regimen results in a high risk of early mortality and often does not eradicate the underlying malignancy *(36,37)*. In 1990, Kolb et al. *(38)* reported that three patients with CML who relapsed after an allogeneic stem cell transplantation achieved a second remission after receiving transfusions of donor lymphocytes. Slavin et al. *(39)* demonstrated that the remissions induced by DLI could be very durable and could be observed in a variety of hematologic malignancies. Posttransplant adoptive immunotherapy has now been used to treat CML, AML, ALL, non-Hodgkin's lymphoma (NHL), myelodysplastic syndrome (MDS), and multiple myeloma. Several conclusions can be drawn from these data:

1. CML patients respond best to this approach, with efficacy being inversely related to tumor burden at the time of relapse. The remission rate for CML patients treated in cytogenetic or early hematologic relapse following stem cell transplantation is approximately 80%, compared with only 12% for patients treated in accelerated phase or blast crisis *(40)*.
2. Response rates for patients with acute leukemia range from only 10 to 20% *(41)*.
3. Preliminary results for patients with MDS and multiple myeloma are encouraging with reported response rates ranging from 25 to 60% *(41,42)*.
4. Most responses are durable, especially in patients with CML; however, late relapses may occur in approximately 40% of patients with other hematologic malignancies *(43)*.

Toxicities associated with DLI have not been trivial and include GvHD and marrow aplasia. The estimated treatment-related mortality rate ranges from 12 to 22% *(40,44,45)*.

There are still many unanswered questions regarding the use of DLI. What is the optimal dose and infusion schedule of T-cells in related and unrelated donor settings? How can the risk of complications such as marrow aplasia and GvHD be minimized? Several clinical trials have been undertaken to provide insight into these unresolved issues. Defining the optimal cell dose and timing for DLI is important, since there appears to be a narrow therapeutic window associated with this form of posttransplant immune modulation. Mackinnon et al. *(46)* reported that a minimum of 1×10^7 CD3+ cells/kg was required to achieve a remission in CML patients, most of whom relapsed after stem cell transplantation from an HLA-identical sibling. Administering 5×10^7 or more CD3+ cells/kg was associated with an increased risk of GvHD.

As might be expected, most studies have correlated lymphocyte dose with the probability and severity of GvHD. For recipients of unrelated donor grafts, infusing a given T-cell dose results in more extensive GvHD than does the infusion of donor lymphocytes from an HLA-matched sibling *(44)*. Consequently, different lymphocyte dosing regimens have been utilized for both related and unrelated donor transplants *(47)*. The optimal T-cell dose for DLI will probably depend not only on the degree of HLA disparity between donor and recipient but also on the disease being targeted, as well as the tumor burden at the time the infusion is initiated. Since these factors vary from patient to patient, the use of intrapatient T-cell dose escalation might minimize the risk of severe GvHD, while retaining the desired antileukemic effect. Escalating the T-cell dose for individual patients also takes advantage of the preclinical observation that multiple infusions of progressively larger numbers of donor-derived T-cells can be given sequentially without causing GvH, whereas a single moderate-sized dose can cause severe GvHD *(48)*. In this regard, Bacigalupo et al. *(45)* reported that relapsed CML patients receiving single-donor leukocyte infusions containing $>1 \times 10^8$ cells/kg had a significantly higher risk of developing GvHD and a lower survival probability than did patients receiving multiple infusions containing fewer cells given in an escalated fashion. A similar observation was made by Dazzi et al. *(47)*, who compared patients receiving a single DLI with those treated with escalating doses of donor lymphocytes administered 12–33 wk apart. The latter group experienced a lower incidence of GvHD, despite receiving the same total lymphocyte dose. The probability of achieving a cytogenetic remission was higher with the escalated dose regimen, although this difference was not statistically significant.

The main complications associated with the use of DLI are bone marrow aplasia and GvHD. Marrow aplasia has been reported in 5–50% of patients treated with DLI, and it contributes to posttransplant morbidity and mortality *(39,40)*. Myelosuppression may be a direct consequence of donor T-cells targeting donor-derived hematopoietic tissue that is presenting passively acquired host antigens, or it may be secondary to inhibitory growth factors released after initiation of GvHD. The risk of aplasia is inversely related to the degree of donor chimerism at the time DLI is instituted. Keil et al. *(49)* reported that of four patients with 40% or more donor CD34+ cells detected in the marrow, none developed aplasia. However, the three patients with <5% CD34+ donor cells all experienced this complication *(49)*. Hence, patients at greatest risk

of developing pancytopenia following DLI are those exhibiting predominantly host hematopoiesis, such as CML patients in hematologic relapse *(50)*. Conversely, early implementation of DLI, prior to loss of donor engraftment (or prior to hematopoietic overgrowth with host-derived malignant cells), minimizes the risk of aplasia. If marrow aplasia occurs, the infusion of donor stem cells often restores hematopoiesis, although this strategy is not always effective.

Perhaps the greatest obstacle limiting the use of DLI is the frequent development of clinically significant GvHD, with approximately 40–70% of patients developing this complication *(39,40,45)*. GvHD, of course, remains a significant problem affecting allogeneic transplant patients in general. Consequently, much attention has been focused on separating GvL from GvHD.

2.2.3. Separating GvL from GvH

To dissociate GvL from GvHD, it must be postulated that different effector cell populations, or at least different cytolytic pathways, are responsible for immune-mediated cell death. Alternatively, it is possible that leukemic cells and normal host tissues are differentially susceptible to the same effector mechanisms. For example, in a murine transplant model, blocking the fas/fas-ligand pathway ameliorated GvHD, without impairing GvL *(51)*. However, when perforin-deficient recipients were utilized or animals were treated with an anti-TNF-α antibody, both GvHD and GvL were attenuated and animals died of progressive leukemia.

Several strategies have been proposed to reduce the risk of GvHD while preserving the GvL effect. These include (1) T-cell depletion with delayed addition of T-cells; (2) T-cell subset depletion; (3) immune modulation with cytokines; (4) ex vivo generation of tumor-specific CTLs; and (5) control of GvHD by activation of a suicide gene. It has long been recognized that T-cell depletion reduces the risk of GvHD in allogeneic transplant recipients. However, any survival advantage gained by attenuating GvHD has generally been nullified by an increased risk of graft rejection, relapse, and fatal viral infections. To overcome these limitations, some investigators have transplanted patients with T-cell-depleted grafts and then infused defined numbers of T-cells within 2 mo of BMT to prevent relapse *(52)* or have administered DLI at the time of a documented relapse *(53)*.

Another approach, previously discussed, has been to administer grafts (or DLI) depleted of T-cell subsets thought to cause GvHD, but not required for the GvL effect. The fact that different T-cell subsets have been targeted for depletion underscores the need to elucidate further the cells responsible for eliciting GvHD and GvL. An alternative strategy to polarize alloreactivity in favor of GvL involves the administration of immunologically active cytokines. Considerable data generated in preclinical murine models suggest that certain cytokines (such as IL-2, IL-11, IL-12, and keratinocyte growth factor) may stimulate GvL activity without potentiating GvHD. IL-2 administration has been shown to protect allogeneic murine transplant recipients from GvHD yet facilitate GvL against a myeloid leukemia *(54)*. However, the effect of IL-2 is probably dependent on the model, as well as the dose and/or schedule of administration, since this cytokine has also been shown to induce fatal GvHD and blunt the GvL response *(55)*. IL-11 has been shown to inhibit GvHD, while retaining both CD4- and CD8-mediated GvL in animal models *(56,57)*. IL-11 also has thrombopoietic activity and may protect the gastrointestinal tract from GvHD-related toxicity, increasing its appeal as a posttransplant immunomodulatory agent. IL-12 has also been shown to inhibit GvHD while preserving a GvL response *(58)*. This effect appears to be at least partly mediated by an IFN-γ-dependent mechanism. Similarly, the administration of keratinocyte growth factor reduced the risk of acute GvHD while preserving CTL activity, proliferation, and IL-2 production in an allogeneic murine transplant model *(59)*. Clinical trials will be required to determine whether these exciting preliminary findings will translate into genuine benefit for allogeneic transplant recipients.

An elegant method to separate GvHD from GvL would be to generate CTLs in vitro that possess specificity against leukemia-specific or leukemia-associated antigens *(60)*. The feasibility of this approach has been demonstrated by producing CTLs against minor histocompatibility antigens expressed exclusively on hematopoietic tissue, as previously discussed *(27)*. CTL tolerance to host antigens has also been obtained by incubating donor lymphocytes with host-derived B-lymphoblastoid cell lines. While in culture, lymphocytes activated by host HLAs can be removed by florescence-activated cell sorting. The resulting T-cell population demonstrates normal helper and cytotoxic responses against foreign antigens but remains anergic to host HLAs.

Finally, rather than trying to dissociate GvHD and GvL, it might be possible to abort the T-cell clone responsible for inducing GvHD after the development of clinical symptoms. This can be accomplished by inserting a "suicide gene" into donor lymphocytes prior to DLI. Bonni et al. *(61)* transduced lymphocytes obtained from allogeneic donors with the herpes simplex thymidine kinase suicide gene, before infusing these cells into recipients who had relapsed or developed Epstein-Barr virus (EBV)-induced posttransplant lympho-proliferative syndrome. Three patients developed GvHD that was controlled by the administration of ganciclovir, which "detonated" the suicide gene and thereby eliminated the transduced cells.

Hence, recognition of the GvL effect has allowed physicians and investigators to develop strategies aimed at improving survival for patients with hematologic malignancies who have undergone allogeneic stem cell transplantation. However, many patients who might benefit from an allogeneic transplant are ineligible for this procedure, because they lack a suitable HLA-compatible donor or have preexisting conditions that preclude the administration of myeloablative therapy. For these individuals, it may still be possible to eradicate malignant cells by exploiting immunologic mechanisms.

3. IMMUNOTHERAPY FOR PATIENTS WITHOUT A STEM CELL ALLOGRAFT

3.1. Cytokines as Potential Antileukemic Therapy

Cells of the immune system communicate with each other through cell–cell contact and soluble intercellular molecules, which are secreted and then activate cellular responses through

interaction with cell-bound receptor molecules. These soluble mediators include a large list of proteins that are made by a variety of cells and have pleiotrophic effects on a variety of tissues, hence their designation as cytokines. A subset of these cytokines, termed interleukins, appears to be made by cells of the immune/inflammatory system and to exert at least some of their effects on cells of this system *(62)*. Some of these interleukins have been shown to have antileukemic potential, either in clinical settings or in experimental models. The first cytokines to be characterized for antileukemic effects were the interferons—molecules that possess diverse antiproliferative effects on certain neoplasms, as well as immunologic and antiangiogenic properties *(63)*. IFN-α has been shown to induce clinically meaningful antileukemic effects in patients with CML and has become a major component of therapy for this disease *(64)*. Its actions are felt to be due to a direct antiproliferative effect on the leukemic myeloid cells. As newer, potentially more effective treatments emerge from clinical trials, the role of interferon in CML therapy will require further evaluation.

IL-2 is the best studied of the interleukins *(65)*. It is a 15-kDa molecule that stimulates NK and T-cells, with less striking effects on B-cells and monocytes. In murine models, treatment with IL-2 in vivo effectively enhanced the antileukemic effect of adoptively transferred T-cells that were specifically able to recognize viral antigenic determinants on the experimental leukemia cells *(66)*. Thus, provision of IL-2 would be a potential component of clinical therapy when leukemia-specific T-cells were identified and infused. Although this may become clinically feasible, identification of leukemia- specific autologous T-cells has been problematic for most forms of human leukemia *(23)*. In addition, infusion of IL-2 alone can activate both T-cell and NK populations in vivo. Thus, infusions of IL-2 were shown to mediate some in vivo protection for leukemia- bearing mice *(67)*. Furthermore, some patients receiving IL-2 for refractory leukemia have shown transient antileukemic effects. For this reason, clinical trials have tested the role of IL-2 following autologous stem cell infusions for patients with AML as well as lymphomas *(68, 69)*. Separately, IL-2 is being tested in a large randomized trial for patients with AML following completion of all scheduled cytotoxic chemotherapy *(69)*. Although these ongoing studies may document a role for effector cell activation with IL-2 in the treatment of leukemia patients, preclinical data suggest that the antitumor efficacy of IL-2 would be enhanced if the effector cells it is activating could be more selectively directed toward recognizing and destroying the malignant cells.

3.2. Monoclonal Antibodies in Leukemia Treatment

The creation and use of monoclonal antibodies (MAbs) able to recognize human leukemia cells has provided a powerful tool for basic cellular and molecular investigations into the workings of the human immune and hematopoietic systems *(70)*. Furthermore, these antibodies are able to provide a selective recognition mechanism that can to identify molecules expressed specifically on a restricted range of normal cells and on the malignant cells derived from them. Thus, MAbs against the CD3, CD4, CD8, CD19, CD20, CD33, and several other surface determinants have been used clinically for a variety of purposes *(70)*, including a vast array of diagnostic tests, as well as in vitro and in vivo treatments. In addition, MAbs have been used in the form of murine-derived monoclonal immunoglobulin. These are genetically engineered structures designed to be less foreign to the human immune system (chimeric, humanized, or fully human MAbs) or as molecular carriers for other agents (i.e., toxins or radionuclides) that have been linked or conjugated to the MAbs *(71)*. This section briefly reviews the use of nonconjugated MAbs; the use of MAbs as immunotoxins and immunoradiopharmaceuticals is summarized by Sievers and coauthors in Chapter 38.

3.2.1. In Vitro Purging

MAbs can be used effectively to eliminate subpopulations of cells from peripheral blood or bone marrow-derived cell preparations. T-cell-depletion with MAbs has been a major technique for T-cell-depleted allogeneic stem cell transplantation *(72)*. A similar technique can eliminate most malignant T-cells from a stem cell preparation by eliminating most T-cells. Clearly, these purging techniques can effectively remove several logs of leukemia cells from a patient-derived stem cell preparation. For several years, many transplant centers pursued autologous transplants with autologous marrow preparations that had been depleted of most leukemic cells, most often (at least for lymphoid malignancies) by using MAb-based purging techniques *(73)*. However, the vast majority of patients treated by this technique were still experiencing leukemic relapses following the autologous transplant procedure.

The high frequency of relapse was felt to reflect three shortcomings in this approach. First, even though the purging techniques were removing most residual leukemia from the infusate, innovative gene- marking studies documented that at least some of the relapsing leukemic cells were derived from the in vitro-purged marrow preparation that had been infused into the patient after "ablative" therapy *(74)*. Second, even if the marrow infusate were devoid of leukemic cells, the high relapse rates observed after transplantation reflect the difficulty in eradicating residual leukemia using conventional ablative regimens (as evidenced by the high relapse rate for the recipients of leukemia-free bone marrow from identical twins) *(75)*. Finally, without some form of immune-mediated antileukemic effect (such as the GvL effect), relapse rates are likely to remain high *(75)*. Thus, although purging of marrow might potentially enhance leukemia-free survival following autologous stem cell infusion, this treatment is not likely to be effective unless a better antileukemic effect can be mounted against the residual leukemia remaining in the patient.

3.2.2. In Vivo Infusion of MAbs

The initial clinical use of antileukemic MAbs in vivo produced what appeared to be dramatic antileukemic effects, but these were short lived. Infusion of anti-common ALL antigen (CALLA) MAbs in patients with CALLA+ ALL resulted in a dramatic and rapid decrease in circulating CALLA+ cells *(70)*, although the number of circulating leukemic cells did not drop as precipitously, and with time the circulating blasts became CALLA+. This striking example demonstrates how some MAbs can modulate expression of cell surface molecules with-

out necessarily destroying the cells. In this example, the leukemic cells had transiently lost their surface expression of CALLA due to the effect of the MAbs, while retaining their viability. This clearly indicates that the MAbs interacted with the leukemic cells and modulated their surface expression of CALLA molecules but were unable to deliver a destructive effect. Thus, for a MAb to show antileukemic efficacy in vivo, it must selectively home to and recognize specific surface antigens on leukemic cells, retain its binding to the cells long enough to deliver a destructive signal, and have the capability to deliver such signals. In the case of immunotoxins, the destructive signal is provided by the toxin or radionuclide attached to the antibody. In some instances, the antibody-toxin conjugate must be internalized by the leukemic cells, whereas for native or genetically engineered MAbs, the killing effect can be delivered, through the induction of apoptosis, receptor blockade, complement-induced lysis, or effector cell-mediated lysis.

To induce apoptosis, the antibody may recognize and bind to a cell surface "death receptor." For example, the MAb to cell surface Fas can induce apoptosis by effective membrane crosslinking of cells that express this receptor *(76)*. However, such death receptors have not been shown to be leukemia-specific, which limits their utility as therapeutic targets. Second, the antibody may bind to an obligate growth factor receptor and prevent stimulatory signaling by the growth factor *(77)*. Although such antibodies have been identified and shown to possess activity in vitro [i.e., MAbs to the IL-2 receptor β-chain blocked cell growth stimulated by IL-2 *(78)*], it is virtually impossible to maintain a sufficiently high concentration of a receptor-blocking MAb in vivo to block all functional receptors fully.

Third, an antibody can utilize the lytic mechanisms of activated human complement to destroy cells that have been bound by the MAb. This mechanism is somewhat limited, as human complement is not a particularly effective pathway for destroying nucleated cells. Even so, certain antibodies have a conformation that makes them particularly good at activating human complement. For example, the Campath-1 MAb, which recognizes the CD52 determinant expressed on virtually all mature B- and T-cells *(79)*, has been used as a potent immunosuppressant in vivo and is now being tested for its antitumor potential against lymphoid malignancies. A recent study has shown that impressive results can be obtained with this MAb in patients with chronic lymphocytic leukemia (CLL). [Of a group of refractory CLL patients, 97% showed rapid elimination of leukemic cells from the blood, with 36% achieving complete bone marrow remissions *(80)*.] However, the vast majority of MAbs are not nearly as effective in activating human complement.

Finally, when the Fab end of an antibody binds to its cognate antigen, the resulting conformational change in the Fc end of some immunoglobulin molecules allows them to bind to and trigger signaling through FcR-III structures expressed on a variety of cells, particularly NK cells, monocytes, and polymorphonuclear cells. Such cells can then induce killing of antibody-coated target cells via a process designated antibody-dependent cellular cytotoxicity (ADCC). In order for this ADCC mechanism to be effective, a sufficient number of activated effector cells need to be available. The number and activation status of these effector cells can be augmented by in vivo treatment with cytokines, particularly those that act on NK cells (like IL-2) and those that act on monocytes, such as GM-CSF and M-CSF *(81)*. Thus studies in murine models have shown that the antitumor efficacy of MAbs can be enhanced by simultaneous treatment with IL-2 or other activating cytokines. Clinical trials testing this concept are under way with MAbs directed against GD2 in patients with neuroblastoma or melanoma *(82,83)*.

Antibodies against hematopoietic malignancies are also being combined with effector activators in clinical trials of this mechanism *(84)*. Further clinical trials of this concept are now being planned with other antibodies reactive against hematopoietic malignancies and known to induce ADCC. The optimal in vivo application of this multimodality treatment strategy remains uncertain. In all likelihood, it would be more effective for eradicating residual leukemia in patients in remission than for inducing remission in patients with a recent relapse. However, if effector cells are required to mediate ADCC, the combined MAb and cytokine treatment would need to be given after rather than concurrently with a course of myelosupressive chemotherapy, to ensure that effector cells are available to mediate ADCC.

3.3. T-Cell Immunity to Autologous Leukemia Through Vaccination

As mentioned earlier, certain molecular targets have been identified on leukemic cells that may allow recognition by T-cells. Unlike allogeneic T-cells, which potentially could target alloantigens for antileukemic immune recognition, autologous "antileukemic" T-cells must identify target structures that are selectively overexpressed on leukemic cells and recognizable by autologous T-cells. In animal models, the best examples of potent "leukemia" antigens seen by syngeneic T-cells are actually foreign antigens; they are the virally controlled antigens expressed by certain leukemia-associated viruses *(66)*. In clinical medicine, only a minority of human hematopoietic malignancies are associated with a viral cause and provide foreign viral molecules that can serve as tumor-specific target antigens *(85)*. Nevertheless, they represent important models, the most instructive being the lymphoproliferative diseases caused by EBV in immunosuppressed patients. Such disorders can be effectively treated by eliminating immunosuppression, so that patients are able to mount an immune response against the viral antigens *(86)*. Activation of autologous T-cells to recognize the strong EBV-associated antigens has also shown clinical efficacy *(87)*. Other viruses associated with hematologic malignancies include the human T-cell leukemia virus family, which has been a target for antiviral T-cell immunity in vitro. Translating this approach into clinically meaningful treatment will require substantial further development.

For the most part, human hematopoietic malignancies are not associated with causative viruses and therefore lack virally controlled foreign antigens. Among the human leukemias, potential antigenic targets include the molecules associated with malignant transformation [such as the BCR-ABL or RARα peptides, discussed above *(24)*]. Another example of a malignancy-associated antigen is the peptide generated in transformed B-cells by

a unique rearrangement of immunoglobulin genes. The resultant alteration in the Fab region is present in every cell in the malignant clone and therefore represents a *bona fide* tumor-specific molecule. The team of Bendandi et al. *(88)* has used this marker effectively as a target for immunotherapy. Patients with B-cell lymphomas had their malignant Fab region idiotype cloned and reproduced in clinical vaccine quantity. Each patient then received a course of standard chemotherapy to induce remission, followed in 6 mo (to allow immune recovery from chemotherapy) by a vaccination regimen consisting of idiotypic protein from the patient's tumor cells, together with injections of GM-CSF to activate antigen-presenting cells. Most patients showed induction of both T- and B-cell-specific antiidiotypic immunity following vaccination, and many entered "molecular remission," based on polymerase chain reaction testing for lymphoma-associated translocation markers.

This impressive example from the study of Bendandi et al. *(88)* suggests that the following conditions need to be in place for vaccine therapy to provide benefit: (1) the antigen must be expressed selectively on tumor cells; (2) the patient's T-cells must be capable of recognizing the tumor antigen; (3) the number of tumor cells present must be small enough to be controlled by a T-cell response and also small enough not to cause global immune suppression; and (4) the T-cell system of the patient must be sufficiently responsive to allow recognition of and expansion by the vaccine regimen. New molecular targets are being sought for similar immune-directed approaches.

4. SUMMARY

Progress in leukemia treatment has provided dramatic advances that have benefited a large number of children with this disease, as well as a growing number of adults. Although the vast majority of patients with acute leukemia achieve complete remission, and many (particularly children) remain in remission and appear to be cured, the specter of recurrent disease continues to affect even the most successful treatment programs. Providing cures for all patients will involve augmenting standard clinical management strategies with the advances in disease detection, molecular staging, and treatment that are summarized in this volume. Improved results may also require the incorporation of additional therapies, particularly those whose toxic effects do not overlap with conventional modalities and whose mechanisms of action will not be blunted by the cross resistance of refractory leukemia cells. Treatments based on immune-mediated antileukemic reactions may qualify in this regard. Some patients receiving allogeneic hematopoietic cells as part of their therapy can expect considerable therapeutic benefit from the GvL effect. Better understanding and control of this reaction may allow wider application of stem cell therapies while minimizing the GvH-associated toxicity linked to the GvL effect. Prospective use of donor lymphocyte infusions is providing a means to achieve this goal. A separate goal is the development of effective immunotherapies for leukemia patients not receiving an allogeneic marrow graft. Promising results with cytokines, antibodies, immunotoxins, and activated T-cells have been obtained in murine models and certain clinical settings. Translating these leads into effective clinical strategies will require more widespread collaboration among laboratory and clinical investigators.

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38 Therapy of Acute Leukemia with Monoclonal Antibodies and Immunoconjugates

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CONTENTS

1. INTRODUCTION

Conventional approaches to the treatment of acute leukemia are often associated with toxic effects due to a lack of specificity for malignant cells. These effects range from alopecia and mucositis to more life-threatening consequences that include cardiotoxicity associated with anthracyclines, central nervous system damage from high-dose cytarabine, and veno-occlusive disease from chemotherapy and radiation regimens used to prepare patients for hematopoietic stem cell transplantation (HSCT). Therapeutic approaches that more specifically target the hemotopoietic sysyem should be safer and, as a result, possibly more effective than current nonspecific chemotherapeutic agents or total-body irradiation (TBI). This chapter focuses on the use of monoclonal antibodies and fusion proteins either as a means of delivering chemotherapy or radiation directly to leukemic cells or, in the setting of HSCT, as a means of more specifically targeting increased doses or radiation to the hematopoietic system.

Because leukemia-specific antigens are quite rare, most targeting approaches using monoclonal antibodies have been directed against normal hematopoietic cell surface antigens that are restricted to leukemic blast cells and their normal counterparts. In this manner, specific antibody binding to nonhematopoietic tissues is avoided. In the history of this field, a variety of iterations have been studied, including unconjugated antibodies, radiolabeled antibodies, and antibodies conjugated to other toxic agents (Fig. 1). Each of these strategies is examined here in the context of both antileukemic therapy and as a component of the preparative regimen for HSCT.

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1.1. Unconjugated Monoclonal Antibodies

Target cells can be eliminated by unconjugated monoclonal antibodies by several mechanisms. In antibody-dependent cellular cytotoxicity (ADCC), tissue macrophages eliminate target cells bound by antibody through binding of the antibody Fc receptor. Although it is probably less significant clinically, the Fc portion of immunoglobulin bound to tumor cells can also induce cell death by complement fixation in complement-dependent cellular cytotoxicity (CDC). Although ADCC has been suggested as a mechanism for the marked non-Hodgkin's lymphoma tumor regressions seen in association with anti-CD20 antibodies *(1)*, recent evidence suggests that ligation of CD20 by antibody can disrupt normal signal transduction events and induce apoptosis without ADCC *(2)*. Clinically, it is likely that both ADCC and disrupted signal transduction contribute to this agent's marked efficacy. Unfortunately, similar approaches in acute leukemia have been less successful despite the success observed with unconjugated antibodies to CD20 in non-Hodgkin's lymphoma and Her-2-neu in breast cancer. It remains possible, however, that unconjugated antibody might have a clinical role in the eradication of low levels of disease (*see* Section 2.2 and Chapter 37 by DeSantes and Sondel).

1.2. Fusion Proteins, Immunoconjugates, and Immunotoxins

Because the use of unconjugated antibody has been associated with limited efficacy in patients with significant leukemic

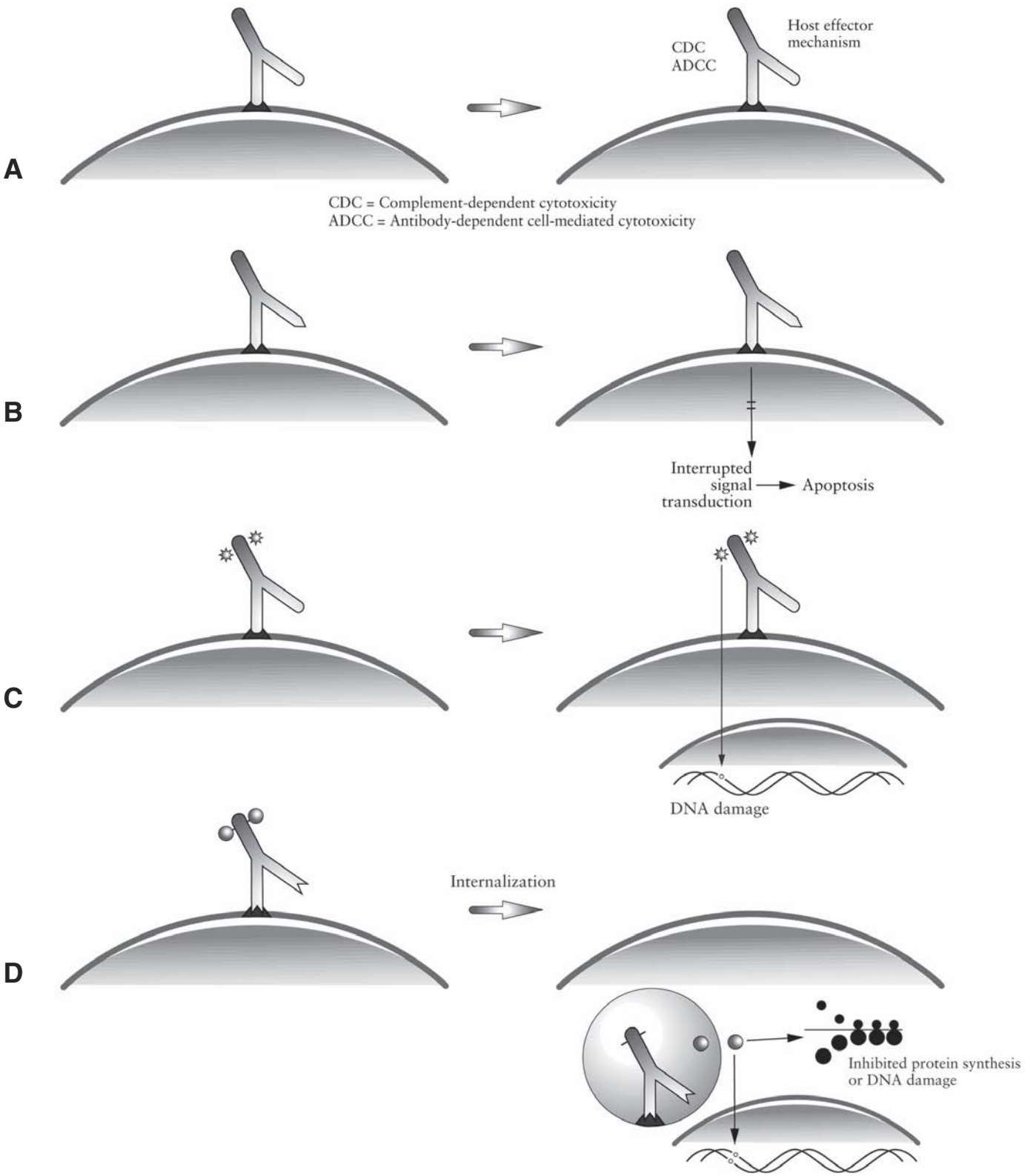

Fig. 1. Simplified mechanisms of antibody-targeted tumor killing. **(A)** Unconjugated monoclonal antibody binding can initiate host-mediated complement-dependent or antibody-dependent cytotoxicity. **(B)** Unconjugated antibody binding can interrupt cellular signal transduction leading to subsequent apoptosis. **(C)** Radiolabeled antibody can remain external when bound to CD45 or become internalized after bonding to the CD33 antigen. Irreparable radiation-induced DNA damage then induces cell death. **(D)** Internalization of the antibody–antigen complex can deliver antibody-bound chemotherapy or toxins to the interior of the cell. Impaired protein synthesis or irreparable double-stranded DNA damage subsequently results in cell death.

tumor burden, humanized monoclonal antibodies have been engineered to deliver cytotoxic agents to cells expressing a particular surface antigen. Similarly, recombinant fusion proteins have been created to present diphtheria toxin specifically to cells that express the human granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor *(3–5)*. Specific tumor targeting should be achieved because normal human myeloid cells, acute myeloid leukemia (AML) cells, and myeloid leukemia cell lines all express this receptor.

Upon binding of an antibody or a fusion protein, some cell surface antigens remain stable on the surface of the cell (e.g., CD45), whereas others internalize and deliver the bound agent to the interior of the cell (e.g., CD33) in a process called modulation. If the cytotoxic agent requires entry to the cytoplasm of the cell to be effective, then selection of a modulating surface antigen is critical for efficacy. Conversely, for certain radioactive isotopes attached to the antibody in such a way that they are cleaved and rapidly excreted from the cell's interior, selection of a cell surface antigen that remains on the cell surface upon binding to antibody may be preferred.

1.3. Radiolabeled Antibodies

Patients with relapsed or refractory AML can achieve long-term remissions after HSCT. However, life-threatening toxic effects are often observed in association with the high doses of TBI and chemotherapy required to obtain durable remissions. Even though leukemic cells are quite susceptible to the DNA-damaging effects of ionizing radiation, systemic toxicity can be dose-limiting. Hence, monoclonal antibodies have been proposed as a means of delivering high doses of radiation to hematopoietic tissues without adversely affecting normal tissues. Radioiodinated antibodies targeting CD33 in the context of AML remission induction therapy have been only partially effective. For this reason, ^{90}Y- and ^{213}Bi-labeled anti-CD33 antibodies have been explored as a means of reducing tumor burden in patients with advanced disease in a nontransplant setting. Newer studies that combine HSCT with radiolabeled antibody targeting the common hematopoietic cell surface antigen CD45 have recently suggested significant clinical efficacy with an acceptable incidence of systemic toxicity.

Randomized prospective clinical studies from the Fred Hutchinson Cancer Research Center (FHCRC) have suggested that the dose of radiation delivered during the HSCT preparative regimen is significantly correlated with the rate of subsequent leukemic relapse. In these studies, patients with AML in first remission *(6)* and chronic myeloid leukemia (CML) in the chronic phase *(7)* received cyclophosphamide and either 12 or 15.75 Gy TBI followed by HLA-matched related marrow transplants. In both studies, the higher TBI dose was associated with a lower risk of subsequent relapse (12% vs 35% for AML; 0% vs 25% for CML). Unfortunately, because the higher TBI dose appeared to contribute to a higher rate of transplant-related mortality, no significant difference in disease-free survival was observed in either study. To circumvent this problem, radiolabeled monoclonal antibodies have been evaluated as a means of increasing doses of radiation to sites of leukemia, including marrow and spleen, without adversely affecting mortality unrelated to relapse.

2. CLINICAL STUDIES

2.1. The Normal Myeloid-Associated Antigen CD33

As pluripotent hematopoietic stem cells mature, they give rise to myeloid progenitor cells with diminished self-renewal capacity and a greater degree of differentiation. During this maturation process, normal myeloid cells express distinct cell surface antigens including CD33 *(8–10)*. Because it is expressed on AML blast cells from about 90% of patients *(9–10)*, the myeloid cell surface antigen CD33 is an attractive target for monoclonal antibody therapy. Although both AML cells and maturing normal hematopoietic cells express the CD33 antigen on the cell surface, normal hematopoietic stem cells lack this antigen *(11)*. Hence, antibody-targeted chemotherapy would probably spare the hematopoietic stem cell. In addition, selective deletion of CD33-positive cells from leukemic marrow aspirates from a subset of patients with AML gave rise to the outgrowth of normal, nonclonal granulocytes and monocytes in a long-term marrow culture system *(12,13)*. These findings suggested that clinical responses might be achieved by selectively targeting and eliminating CD33-positive cells.

2.2 Unconjugated Anti-CD33 Antibody

Attempts to ablate CD33-positive cells selectively in patients were first reported by investigators from Memorial Sloan-Kettering Cancer Center (MSKCC). After intravenous administration of approximately 5 mg/m^2 of trace radioiodinated murine anti-CD33 (M-195) antibody, leukemic blast cells in peripheral blood and marrow rapidly became saturated with antibody, but no significant clinical responses were observed *(14)*. Subsequently, using a strategy designed to optimize ADCC, MSKCC investigators have evaluated supersaturating doses (12–36 mg/m^2/d on d 1–4, then repeated on d 15–18) of unconjugated humanized antibody to the CD33 antigen (HuM-195) *(15)*. Among 10 patients with relapsed or refractory myeloid leukemias (9 AML and 1 CML) treated on the study, 1 achieved a complete remission, 3 had a decrease in leukemic burden, 5 had progressive disease, and 1 was inevaluable for treatment response. Before receiving the anti-CD33 monoclonal antibody, the one patient who achieved complete response had a low level of leukemia (8% blasts) in his bone marrow following two rounds of conventional induction chemotherapy.

In a successive study, 35 patients were treated with either 12 or 36 mg/m^2 of HuM-195 daily for 4 consecutive days weekly for a total of four courses *(16)*. Complete remissions were reported in 2 patients, both of whom were among the 15 patients who had a relatively low leukemic burden pretreatment (<30% blasts in their marrow). Given that clinical responses were observed in occasional patients with low tumor burdens, and the therapy was not associated with significant clinical toxicity, use of an unconjugated antibody might find a role in consolidation of patients with minimal residual disease. Because trials that combined either Herceptin or Rituxan with conventional chemotherapy documented augmented clinical efficacy, analogous combinations of uncon-jugated anti-CD33 antibody with conventional chemotherapy might result in improved response rates.

Unconjugated anti-CD33 antibody has been proposed as a means of consolidating patients with acute promyelocytic leukemia (APL) who harbor evidence of minimal residual disease by reverse transcription-polymerase chain reaction (RT-PCR) analysis *(17)*. In a pilot study, APL patients who achieved complete remission after induction therapy with all-*trans*-retinoic acid (ATRA) received HuM-195 at a dose of 3 mg/m^2 twice weekly for 3 wk. The cohort then received consolidation therapy with idarubicin and cytarabine and an additional 6 mo of monthly maintenance therapy with HuM-195 given in two doses separated by 3 or 4 d. Patients' bone marrow aspirates were evaluated serially for promyelocytic leukemia-retinoic acid receptor α (*PML-RAR*α) mRNA by RT-PCR, and patients were considered evaluable for response if adequate RNA samples were obtained for RT-PCR analysis. Although only 1 of 21 patients was PCR-negative after ATRA alone, an additional 7 of 18 evaluable patients became PCR-negative after receiving the first 3-wk course of HuM-195. Patients subsequently received consolidation with conventional chemotherapy. After this therapy, all 19 evaluable patients lacked evidence of leukemia by RT-PCR. Because it is not known how quickly the *PML-RAR*α fusion gene disappears after ATRA therapy, and because a considerable number of patients enter molecular remission after ATRA alone, it is conceivable that the seven additional patients might have achieved negative PCR results without having received HuM-195 treatment. Nonetheless, these results suggest a potential role for unconjugated anti-CD33 antibody therapy in the consolidation phase of treatment, particularly among patients with evidence of minimal residual disease.

2.3. Conjugated Anti-CD33 Antibody

As noted in the radiolabeled antibody studies described above, administration of anti-CD33 antibody results in saturation of CD33 sites throughout the body and rapid subsequent internalization (modulation) of the antigen–antibody complex by the cell. In an effort to exploit this phenomenon, a potent antitumor antibiotic, calicheamicin, was linked to a humanized anti-CD33 antibody to create the novel agent gemtuzumab ozogamicin (Mylotarg™) *(18)*. In collaboration with Wyeth-Ayerst Research, a phase I study of this agent was conducted in which patients with relapsed or refractory CD33+ AML were treated with escalating doses of drug every 2 wk for 3 doses *(19)*. Of 40 evaluable patients, leukemia was eliminated from the blood and marrow of eight (20%) and blood counts normalized in three (8%) patients. Doses of up to 9 mg/m^2 of gemtuzumab ozogamicin were generally well tolerated. Approximately two-thirds of patients experienced a postinfusion syndrome of fever and chills. Modest and reversible hepatic transaminase elevations and hyperbilirubinemia were occasionally observed in some patients who received gemtuzumab ozogamicin at high dose levels.

Given the promising results of the phase I study, several phase II studies were performed in which patients with AML in first untreated relapse after a period of at least 3 mo were treated at a dose of 9 mg/m^2 every 2 wk for two doses *(20)*. Among the 142 patients, 30% achieved a remission characterized by ≤5% blasts in the bone marrow, ≥1500 neutrophils/mm^3, and platelet transfusion independence. Remission patients were offered autologous or allogeneic HSCT, consolidation chemotherapy consisting of mitoxantrone and etoposide, or no further therapy; >50% remained alive without leukemia for at least 1 yr after the achievement of remission. Because the drug conjugate eliminates normal myeloid and megakaryocytic precursors, severe neutropenia and thrombocytopenia were almost invariably observed. Grade 3 or 4 mucositis (per the Common Toxicity Criteria, Version 1, National Cancer Institute) was observed in 4% of patients, and 28% of patients developed grade 3 or 4 infection. In addition, grade 3 or 4 bilirubin or hepatic transaminase elevations were observed in 23 and 18% of patients, respectively. Median hospitalization for the entire group was 24 d, and 16% of patients were hospitalized for ≤1 wk. Unlike other studies in which numerous immune responses occurred following infusion of immunoconjugates containing either murine-derived monoclonal antibodies or naturally occurring toxins, no patient in the phase II study had a detectable immune response.

2.4. Radiolabeled Anti-CD33 Antibody Followed by HSCT

In two phase I studies, ^{131}I-labeled anti-CD33 has been combined with conventional transplant preparative regimens for patients with advanced AML. With an 8-d half-life and a path length of 0.8 mm, this radioisotope offers the potential of killing antigen-negative cells, or cells not otherwise binding antibody, that are near cells bound by antibody (the *bystander effect*). As a follow-up to initial biodistribution studies demonstrating localization of ^{131}I-M195 antibody in patients with advanced AML, investigators at MSKCC treated nine patients with AML using M195 antibody labeled with 120–160 mCi ^{131}I in divided doses, followed by busulfan and cyclophosphamide and matched related allogeneic HSCT *(21)*. The toxicity of the transplant procedure was not measurably increased by the addition of radiolabeled antibody to busulfan and cyclophosphamide in that group of patients.

The biodistribution of an alternative murine anti-CD33 antibody, p67, was determined at the FHCRC in nine patients with AML beyond first remission or refractory to conventional therapy *(22)*. Four of nine patients had favorable biodistribution of trace-labeled ^{131}I-p67 antibody, with higher estimated radiation doses to bone marrow and spleen as compared with liver, lung, or kidney. These four patients were treated with p67 antibody labeled with the amount of ^{131}I estimated to deliver 1.75 Gy to the normal organ receiving the highest dose, followed by cyclophosphamide, 12 Gy TBI, and matched related or autologous marrow. The therapy was well tolerated, but three of these four leukemia patients relapsed post transplantation. Because of the limited expression of CD33 antigen, the optimum antibody dose for both p67 and M195 antibodies was small (≤5 mg/m^2), with higher, supersaturing antibody doses leading to excess radiation delivery to nontarget organs from prolonged circulation of unbound radiolabeled antibody. In addition, for ^{131}I-p67 antibody, the radiation dose to marrow was limited by the rapid excretion of ^{131}I from target cells after internalization of the ^{131}I-labeled anti-CD33 antibody-antigen complex. Combined with the small dose of either anti-CD33

antibody that could be administered without saturating CD33 sites, this limitation has seriously curtailed dose escalation, leading both institutions to pursue other strategies.

2.5. Radiolabeled Anti-CD33 Antibody Without HSCT

Investigators at MSKCC have labeled anti-CD33 antibody with two alternative isotopes, ^{213}Bi and ^{90}Y, in nontransplant studies in patients with advanced AML *(23,24)*. Bismuth-213 emits an α-particle with a very short path length (0.06 mm) and a 46-min half-life. Although the very short path length offers the potential to minimize nonspecific cytotoxicity, the half-life requires rapid access to target cells for efficacy. In a phase I study, 17 patients with relapsed (n = 13) or refractory (n = 3) AML and 1 patient with chronic myelomonocytic leukemia received escalating doses (0.28–1.0 mCi/kg) of ^{213}Bi-HuM-195 in three to six fractions over 2–4 d *(24)*. Uptake of radioisotope in bone marrow, liver, and spleen was documented by a γ-camera within 10 min of administration, with much higher estimated radiation doses to those tissues compared with nontarget organs and the whole body. Myelosuppression lasting from 8 to 34 d was also observed and was most prolonged at higher isotope doses. No complete remissions had been documented at the time of the report, although reductions in peripheral blood leukemic blast cell counts were seen in 10 of 12 evaluable patients, and 12 of 17 patients had reductions in the percentages of marrow blasts. Given the extent of myelosuppression resulting from this agent, its optimal utility may be in the setting of stem cell rescue.

These investigators have also conducted a phase I study of HuM-195 anti-CD33 antibody labeled with the β-emitting isotope ^{90}Y, which has a half-life of 2.5 d and a path length of 5.3 mm *(23)*. In a nontransplant phase I study, 17 patients with relapsed or refractory AML with a median age of 57 yr received 0.1–0.3 mCi/kg of ^{90}Y-HuM-195. As the absence of γ emissions prevented direct determination of the bio-distribution of ^{90}Y, the agent was combined with trace indium-111-labeled antibody in nine patients. Rigors, fevers, and nausea were commonly noted acute toxic effects. Transient hepatic transaminase abnormalities were observed in 10 patients. The extent of myelosuppression was higher at the highest ^{90}Y dose, and one patient died with marrow aplasia 6 wk after therapy. One patient treated at the highest dose level achieved a complete remission, and 12 of 16 evaluable patients had reduced percentages of marrow blast cells in comparison with pretreatment levels. As with ^{213}Bi-labeled anti-CD33 antibody, higher doses of radiation therapy could be delivered in the setting of stem cell rescue, and such a trial combining ^{90}Y-HuM-195 antibody with etoposide followed by autologous HSCT is planned.

2.5. The Normal Hematopoietic Cell Antigen CD45

In contrast to the CD33 antigen, the CD45 cell surface antigen is densely expressed (200,000 copies per cell) by essentially all white blood cells and their precursors. Virtually all AMLs and most acute lymphoblastic leukemias (ALLs) express the antigen. Because it is present on lymphoid as well as myeloid cells, radiolabeled anti-CD45 antibody can deliver radiation to sites of leukemic involvement in lymph nodes in addition to the marrow and spleen. Furthermore, its expression by most cells in these tissues allows the use of radiolabeled anti-CD45 antibody, whether patients are in remission or relapse. Unlike the CD33 antigen, the CD45 antigen remains stable on the cell surface after antibody binding *(25)*. Thus, although CD33 may represent an excellent target for antibody-drug conjugates since it internalizes upon antibody binding, the lack of internalization and of antibody-bound CD45 antigen provides an advantage when using ^{131}I-labeled antibody, as dehalogenation with rapid clearance of iodine from target sites should be minimized.

2.6. Radiolabeled Anti-CD45 Antibody Followed by HSCT

Investigators at the FHCRC have examined the biodistribution of ^{131}I-labeled BC8 (murine anti-CD45) antibody in patients with AML, myelodysplastic syndrome (MDS), and ALL, and have combined this agent with conventional marrow transplant preparative regimens in patients with advanced disease and with AML in first remission. In a phase I study estimating the amount of radiation that could be delivered by ^{131}I-BC8 antibody, combined with cyclophosphamide and 12 Gy TBI followed by matched related or autologous HSCT, 44 patients with high-risk acute leukemias (including 3 with advanced MDS) received a biodistribution dose of 0.5 mg/kg trace ^{131}I-labeled antibody *(26)*. Thirty-four patients with higher estimated radiation doses to the marrow and spleen than to the liver, lung, or kidney ("favorable biodistribution") received a therapy dose of BC8 antibody labeled with the amount of ^{131}I estimated to deliver from 3.5 Gy to a maximum of 12.25 Gy to the normal organ receiving the highest dose (Fig. 2). Eighty-four percent of patients had a higher amount of radiation delivered to the marrow and spleen than to the liver, the normal organ receiving the highest dose in all but one patient, a much higher rate of favorable biodistribution than was seen with ^{131}I-labeled p67 (anti-CD33) antibody. The highest estimated radiation doses to the marrow were seen in patients with AML in relapse owing to both higher initial uptake of antibody and longer retention of ^{131}I in that tissue. The investigators also demonstrated that administration of a preclearing dose of cold antibody before ^{131}I-labeled anti-CD45 did not significantly reduce hepatic radiation exposure.

The maximum tolerated dose of radiation delivered by ^{131}I-BC8 antibody was estimated to be 10.5 Gy, with grade III regimen-related toxicity (veno-occlusive disease of the liver) in one of six patients treated at this level. Both patients receiving 12.25 Gy developed life-threatening mucositis. Estimated marrow doses of up to 28 Gy were tolerated without impairing engraftment. However, a single patient receiving an estimated marrow dose of 31 Gy failed to engraft prior to her death on d 29 from fungal pneumonia, raising the possibility that excessive radiation to marrow stroma could adversely affect the growth of transplanted hematopoietic cells. Ten of the 34 patients with advanced leukemia or MDS receiving a therapy dose of ^{131}I-BC8 antibody on this study are surviving disease-free 33–107 mo post transplantation. Based on the average estimated radiation doses delivered per mCi ^{131}I, an average of 24 Gy can be delivered to the marrow, and 50 Gy to

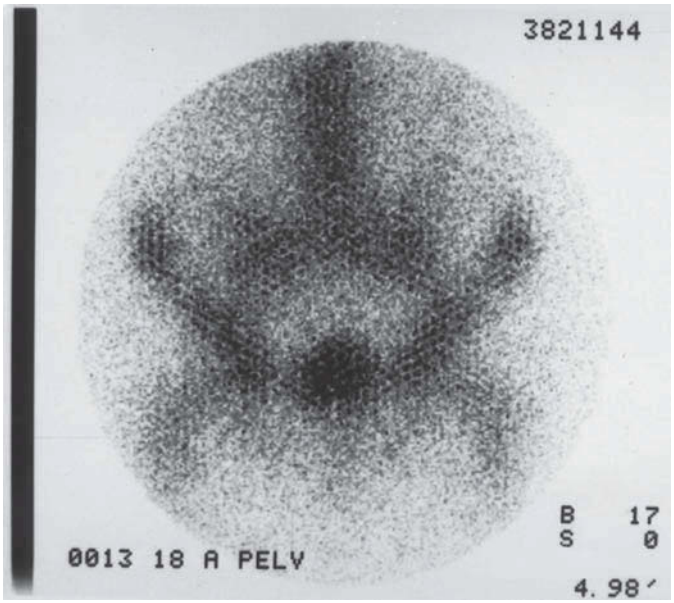

Fig. 2. ^{131}I-anti-CD45 antibody localization. Anterior γ-camera image of pelvis 18 h after ^{131}I-BC8 infusion in a patient with AML in relapse.

the spleen, at the maximum tolerated dose of 10.5 Gy to liver. Phase II studies of this preparative regimen for patients with advanced AML or MDS are under way.

In a similar study, ^{131}I-labeled anti-CD45 antibody has been combined with busulfan and cyclophosphamide in patients with AML in first remission receiving a matched related HSCT *(26,27)*. Favorable biodistribution has been seen in 90% of patients, and 24 patients have been treated with BC8 antibody labeled with the amount of ^{131}I estimated to deliver 3.5 (n = 3) to 5.25 (n = 20) Gy to the liver, an average of 10 Gy to the marrow, and 26 Gy to the spleen. Eighteen of the 24 patients with AML are surviving disease-free for 13–66 mo (median, 45 mo) after transplantation. Four patients died from transplant-related causes, and there have been two relapses among the 20 patients evaluable for relapse. This low relapse rate is encouraging given that one-third of the patients were in an unfavorable risk group based on cytogenetics at diagnosis, with only one patient in a favorable cytogenetic risk group. Historically, approx 30% of patients with AML conditioned with busulfan and cyclophosphamide and transplanted in first remission would be expected to experience leukemic relapse, and posttransplant relapse rates as high as 45–60% have been seen in patients with unfavorable cytogenetics. Should further experience with this regimen confirm these initial results, a phase III study comparing this regimen with busulfan and cyclophosphamide alone would be warranted to determine whether the addition of targeted hematopoietic irradiation to a conventional transplant preparative regimen can decrease the relapse rate without increasing toxicity.

2.7. Granulocyte/Macrophage Colony-Stimulating Factor

The receptor for GM-CSF is expressed by normal monocytes, granulocytes, and macrophages and their progenitors, and on AML cells *(28,29)*. Because endocytosis of bound GM-CSF receptor occurs more rapidly in leukemic cells than in normal cells, GM-CSF also represents an ideal delivery agent for toxins that require intracellular targeting *(29)*. One such agent, diphtheria toxin (DT), induces cell death by inactivation of protein synthesis *(30)*. Intracellularly, the DT is cleaved, the A fragment (the catalytic domain) irreversibly inhibits protein synthesis by enzymatic ribosylation of elongation factor 2, and cell death ensues. Several investigators have attached GM-CSF to the DT catalytic and translocation domains *(3–5)*. The DT-GM-CSF fusion protein was toxic to AML cell lines and AML progenitors but not to normal hematopoietic progenitors.

Because DT-GM-CSF was associated with both significant clinical activity and few toxic effects in a murine model *(31)*, a phase I clinical study of the agent was performed at Wake Forest University and the University of Kentucky *(32)*. Twenty-two patients with relapsed or refractory AML were treated with 15-min intravenous infusions of escalating doses of the fusion protein, from 1 to 5 μg/kg/d for 5 d. Immune responses were observed in 4 of 20 evaluable patients after 30 d. Infusion-related toxic effects included transient fever, chills, nausea, hypoxemia, and hypotension. Several patients also experienced asymptomatic, transient elevations of hepatic transaminases, creatinine kinase, and lactate dehydrogenase. Clinically, reductions in the numbers of leukemic cells in the blood and marrow were observed by d 12 after administration of 4 and 5 μg/kg/d. Although all patients treated with these relatively low doses of DT-GM-CSF ultimately experienced disease progression, the modest antileukemic responses observed are encouraging, and continued dose escalation is planned.

2.8. The Normal Lymphoid Cell Antigen CD19

Normally expressed by B-lineage lymphocytes, CD19 is also expressed by leukemic blasts of most patients with ALL. Like CD33 and CD45, CD19 is not found in nonhematopoietic tissues. The Src family protein tyrosine kinase (PTK), involved with signal transduction and apoptosis, is physically linked to CD19 and serves as an ideal target for the delivery of a toxin or chemotherapeutic agent. Uckun et al. *(33)* at the Parker Hughes Cancer Center have targeted the membrane-associated CD19-Src family PTK complex with B43-genistein immunoconjugate as a means of triggering apoptotic cell death. Genistein, a naturally occurring PTK inhibitor, linked to a monoclonal antibody directed against CD19 (B43), has been found to be highly specific and toxic for human leukemic cells in a SCID mouse model *(34)*. In a phase I clinical study, 15 patients aged 4–60 yr with advanced CD19+ B-lineage ALL (and one patient with chronic lymphocytic leukemia) received escalating doses of B43-genistein as a 1-h intravenous infusion at 0.1–0.32 mg/kg/d dose levels for 10 consecutive d or 3 consecutive d weekly for a total of nine doses. The immunoconjugate was generally well tolerated, and the most common infusion-related adverse event was fever. Grade 2 vascular leak syndrome was observed in one patient. Of nine patients evaluated on d 28 for human antimouse antibody, three had moderately high levels, ranging from 20 to 87 ng/mL. Levels of the immunoconjugate were undetectable after these three patients received a second course of the immunoconjugate, demonstrating rapid clearance of B43-genistein from the circulation due to an immune response. Among the 16 patients treated, 1 of 14 evaluable ALL patients

achieved a complete remission characterized by the absence of leukemic blasts in the bone marrow aspirate and recovery of normal blood counts. Another two patients experienced reductions in leukemic burden as assessed by percentages of blasts in sequential bone marrow aspirates. The immunoconjugate was associated with few clinically significant toxic effects and resulted in a remission of 19 mo in one instance.

3. UNANSWERED QUESTIONS FOR FUTURE STUDY

Although significant progress has been made in the development of monoclonal antibody-based treatments for AML, many questions remain. Phase II and III studies to determine the relative efficacy and toxicities of each approach should help in determining which clinical settings are best suited for these agents. In addition, these studies should help determine when in the patient's overall treatment course drug-antibody conjugates and radiolabeled antibodies should be used.

3.1. Immunoconjugates

First, although significant reductions in tumor burden were observed in most patients with relapsed AML who received gemtuzumab ozogamicin in phase II trials, approximately two-thirds of the patients failed to enter complete remission. Because saturating levels of anti-CD33 antibody were consistently achieved at a dose of 9 mg/m^2, other drug resistance mechanisms are likely to be responsible for treatment failures. In the phase I study, elimination of leukemia appeared to be correlated with a low capacity by leukemic blast cells to extrude the dye $DiOC_2$. Because calicheamicin is a known substrate of the drug efflux pump P-glycoprotein, rapid efflux of calicheamicin from the cell interior might prevent free radicals formed by the agent from irreversibly damaging DNA and producing cell death. If this hypothesis is correct, use of an inhibitor of P-glycoprotein (e.g., cyclosporine) in combination with gemtuzumab ozogamicin might improve the remission induction rate.

Second, several studies that have characterized human AML precursors have suggested that leukemia progenitor cells lack the CD33 antigen *(35,36)*. However, it remains difficult to reconcile the fact that about a third of patients who received gemtuzumab ozogamicin in phase II studies appeared to achieve both remission and subsequent restoration of normal hematopoiesis by remaining CD33$^-$ precursors. In these patients, the CD33$^-$ precursors might indeed be predominantly or completely nonmalignant. This hypothesis is supported by findings that, in some cases of AML, the clonal abnormality originated in either a committed progenitor or an early multipotent cell whose proliferative expression is mainly restricted to the granulocyte/monocyte lineage *(37)*. Because selection of CD33$^-$ progenitors from some of these leukemias enabled normal hematopoietic growth in culture, the malignant clone may involve few, if any, CD33$^-$ precursors *(12,13)*.

In other patients with AML, and possibly those who failed to respond to gemtuzumab ozogamicin, the clonal abnormality has been found in both the erythroid and myeloid lineages, suggesting that multipotent precursors are involved in the malignant process *(37)*. Might normal hematopoiesis also be restored in these patients even if a substantial portion of CD33$^-$ precursors were malignant because normal precursors express at least a short-term proliferative advantage? Although this account appears to contradict observations on leukemic growth patterns in a immunodeficient murine model after administration of primitive (CD34$^+$ CD38$^-$) precursors from human leukemia samples *(38)*, it is consistent with the report that a patient undergoing allogeneic HSCT who was inadvertently given an infusion of donor AML cells initially recovered with normal donor hematopoiesis before ultimately manifesting AML *(39)*. In patients with multipotent AML, tumor reduction might be achieved by the elimination of CD33$^+$ myeloblasts, although allogeneic HSCT might ultimately be required to obtain durable remissions.

3.2. Radiolabeled Antibody Conjugates

Drug-antibody conjugates such as gemtuzumab ozogamicin have the advantage of not binding to hematopoietic stem cells, so that rescue with HSCT is not mandatory. However, because internalization of the toxic moiety into a cell is required for cell kill, the antibody must bind to all cells of interest. In contrast, the bystander effect provided by the path length of isotopes conjugated to antibody results in the delivery of radiation to hematopoietic stem cells, even if those cells do not express the target antigen. Thus, the delivery of maximum doses of radiation to leukemic cells by radiolabeled antibody requires HSCT rescue. However, this approach offers the advantage of exposing leukemic cells to radiation even when some do not express antigen, or when the patient is in clinical remission and the antibody is binding predominantly to normal hematopoietic cells. It is conceivable that remission induction using antibody-targeted chemotherapy followed by consolidation with radiolabeled antibody will achieve long-term remissions while minimizing toxic effects. Several patients in Seattle with recurrent AML have received gemtuzumab ozogamicin to induce a second remission and then received HSCT following a preparative regimen that incorporates ^{131}I-labeled anti-CD45 antibody.

For radiolabeled antibody, there is controversy regarding the optimal isotope for clinical use. There are decades of experience with the use of ^{131}I as a therapeutic agent, and the radiolabeling chemistry is straightforward and reliable. The γ-component allows for direct determination of antibody biodistribution in patients using quantitative γ-imaging and contributes a component of "TBI" that may be therapeutic in patients with "liquid tumors" such as leukemia. However, because of the γ-component, patients receiving antibody labeled with large amounts of ^{131}I must be treated in radiation isolation rooms. In contrast, the absence of a γ-component for ^{90}Y allows the treatment of patients in the outpatient setting and results in much less radiation exposure to staff and family members.

However, the direct determination of antibody biodistribution in a patient requires that the antibody be labeled with the surrogate isotope ^{111}In, which can be imaged by a γ-camera. The ability to extrapolate from the biodistribution of ^{111}In-labeled antibody to that of ^{90}Y-labeled antibody has been hampered by the findings that ^{111}In and ^{90}Y do not always bind equally tightly within a given chelate

structure bound to an antibody and that the distribution of *free* ^{111}In and ^{90}Y differ. The path length of the β-component of isotopes will also theoretically affect the delivery of radiation at a microscopic level. An isotope with a longer path length, such as the 5.3 mm provided by ^{90}Y, will result in greater homogeneity of radiation delivery within a tissue when the antibody binding is somewhat heterogeneous, compared with the 0.8 mm of ^{131}I. However, for small clumps of cells, a greater proportion of the radiation delivered by isotope bound to those cells will be delivered to the cells using isotopes with a shorter path length.

There is much less experience with α-emitting isotopes, in part because the labeling chemistry is complex, the isotopes are not readily available, and the short half-life requires a setting in which the radiolabeled antibody can reach target cells quickly. Given that the malignant cells of acute leukemia reside in tissues such as the marrow and spleen that have a high blood flow, acute leukemia is probably the best clinical setting in which to test the efficacy of such radioimmunoconjugates. Furthermore, because of the very high energy of these particles, cell kill can result from trangression of the cell by only a few particles, and there is theoretically less chance that a cell may repair sublethal damage.

Because of the many physical and chemical differences between radioisotopes and radioimmunoconjugates that may influence their biologic effect, it will be a challenge to determine which will provide the best clinical efficacy with the least toxicity. Current and future clinical studies will require careful analysis of the estimated ratios of radiation delivered to target compared with nontarget tissues, as well as the correlation between estimated radiation doses delivered and the resulting biologic outcomes, including both the intended antileukemic effect and the associated toxicities.

4. CONCLUSIONS

Unconjugated humanized anti-CD33 antibody appears able to ablate a limited number of tumor cells from patients with AML and may prove to have a role in the treatment of patients with minimal residual disease. As a means of improving the ability of antibodies to eliminate tumor, potent cytotoxic agents or radioisotopes have been conjugated to monoclonal antibodies to increase targeted cell killing. Humanized anti-CD33 antibody linked to the potent antitumor antibiotic calicheamicin (gemtuzumab ozogamacin) used as a single agent has safely induced remission in one-third of patients with AML in first relapse. Also, in the non-HSCT setting, HuM-195 (anti-CD33) antibody labeled with either ^{213}Bi or ^{90}Y has reduced tumor burden in patients with relapsed and refractory AML without significant nonhematologic toxicity.

As a method of specifically targeting radiation to hematopoietic organs as part of an HSCT preparative regimen, ^{131}I-labeled anti-CD45 antibody has been shown to deliver a higher dose of radiation to the bone marrow and spleen than to other vital organs. There appears to be significant clinical efficacy with this approach. Only 2 of 24 patients with AML in first remission treated in this manner experienced recurrent leukemia. Ongoing studies of these monoclonal antibody-based therapies will better define their efficacy and optimum use.

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VI DRUG RESISTANCE

39 Drug Resistance in Acute Leukemias

JEAN-PIERRE MARIE AND OLLIVIER LEGRAND

CONTENTS

1. INTRODUCTION

Drug resistance in acute leukemia was first described by Farber et al. *(1)* in cases of acute lymphoblastic leukemia (ALL) and is still the major cause of death in all types of acute leukemia. A mathematical model for the development of drug resistance in tumors was proposed in 1979 by Goldie and Coldman *(2)*, based on the hypothesis that cancer cells have a high spontaneous mutation rate that leads, over time, to the emergence of cells resistant to chemotherapeutic drugs. To reduce the rate of emergence of resistant cells according to this hypothesis, the simultaneous administration of multiple drugs with different targets was suggested. Despite the introduction of combination chemotherapies, treatment failures continued to be observed. Rates of initial treatment failure and relapse were lowest in childhood ALL, whereas adults with ALL or acute myeloid leukemia (AML) mainly died of their disease, regardless of the treatment they received. Over the last 20 years, experimental models and clinical research have identified several causes of drug resistance in tumors. This improved understanding of the mechanisms involved in drug resistance has permitted the development of new therapeutic strategies.

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2. MEASUREMENT OF DRUG RESISTANCE IN CLINICAL SAMPLES

In vitro assays have been developed to test the drug resistance of clinical samples *(3,4)*. Clonogenic assays offer the advantage of testing the drug sensitivity of leukemic progenitor cells and have been used to show that resistance to an anthracycline and cytarabine is highly correlated with clinical failure in adult AML and that such resistance has independent significance in multivariate analysis *(4)*. Unfortunately, these clonogenic assays are time-consuming and difficult to automate, leading to the development of other in vitro tests. The most commonly used of these is the methyl-thiazol-tetrazolium (MTT) assay, which relies on the ability of the mitochondria of living cells to convert a soluble tetrazolium salt (MTT) into an insoluble formazan. The formazan precipitate is purple and can be dissolved; its extinction can be read on a 96-well plaque reader. The extinction is linearly correlated with the number of viable cells in suspension. The 4-d MTT assay is an efficient tool for large-scale drug-resistance testing, and results showed a good correlation with prognosis in childhood ALL *(3,5)* and adult AML *(6)*.

3. CLASSIFICATION OF DRUG RESISTANCE

The efficacy of cytostatic antineoplastic therapy is determined by a sequential cascade of events, including drug delivery, drug-target interaction, and the induction of cellular

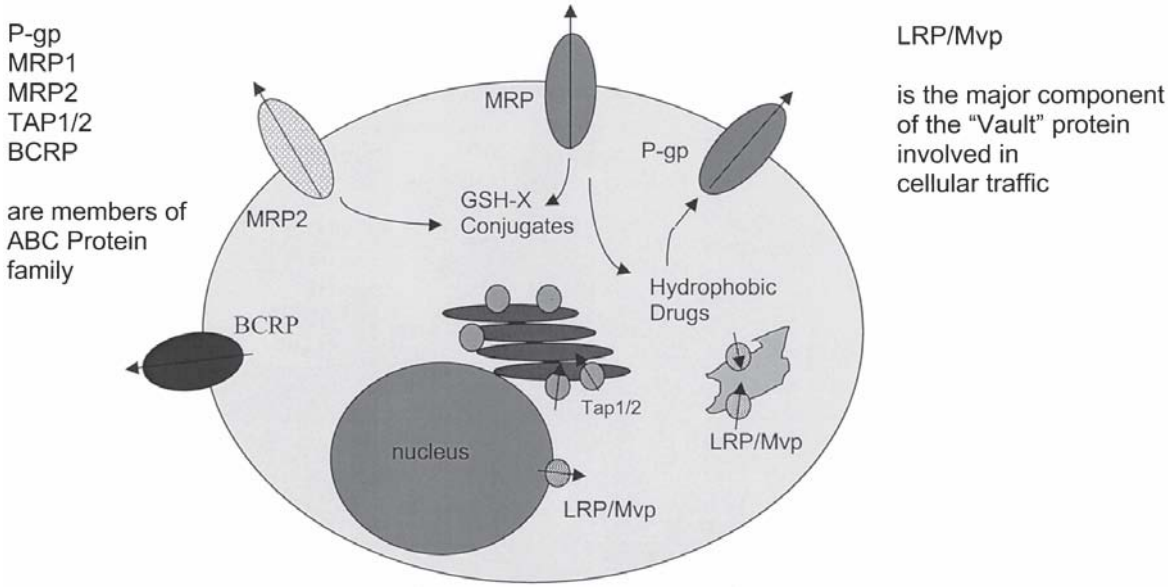

Fig. 1. Transport mechanisms involved in cellular drug resistance. Several ATP binding cassette (ABC) proteins are involved in transport of xenobiotics out of the cells; P-glycoprotein (Pgp), the multidrug resistance-associated protein (MRP) family, and breast cancer resistance protein (BCRP) are part of the cytoplasmic membrane. Transporter associated with antigen processing (TAP) is expressed on the endoplasmic reticulum membrane. Lung resistance protein/major vault protein (LRP/MVP) expression is correlated with multidrug resistance in non-Pgp cell lines, but until now cDNA transfection/antisense inhibition has failed to demonstrate a direct correlation between LRP/MVP expression and drug resistance. GSH-X, reduced glutathione.

Table 1
Drug Resistance Mechanisms

Before reaching the target	*During or after reaching the target*
Pharmacology	Drug target alteration
Host drug metabolism	Increased or decreased target level
Tumor drug delivery	Altered function of target
Cellular transport	DNA repair
Decreased drug influx	Increased DNA repair
Increased drug efflux	No recognition of DNA alteration
Cellular metabolism	Apoptosis and cell cycle
Decreased drug activation	Altered function
Increased drug catabolism	Altered regulation

damage. The first part of this cascade corresponds to pharmacologic resistance and has been the most widely studied mechanism of resistance.

Classically, drug resistance has been divided into extrinsic and intrinsic causes (Table 1). *Extrinsic resistance* corresponds to the inability of the drug to reach the tumor cell; this is the case when the bioavailability of the oral form could vary greatly from patient to patient, as with 6-mercaptopurine in ALL *(7)*. *Intrinsic resistance* is directly due to the properties of the tumor cell. This phenomenon can be observed in vitro and can be classified as *simple resistance* (when the cells are resistant to only one drug) or as *multidrug resistance* (when a cross resistance is observed among chemostatic drugs with different targets). The latter type of resistance, which is largely observed in the clinic, can be attributed to several mechanisms.

4. INCREASED DRUG EFFLUX: ABC PROTEINS

Multidrug-resistant (MDR) cells are resistant to several naturally occurring plant or microbial products, but not to synthetic compounds, such as nucleotide analogs like cytarabine. The resistant tumor cells maintain lower intracellular drug concentrations than do their sensitive counterparts *(8)*, and in the large majority of cases express transport proteins of the ATP Binding Cassette (ABC) superfamily, responsible for active efflux of the drugs *(9)*.

P-glycoprotein (Pgp), the first ABC protein described by Juliano and Ling in 1976 *(10)* and multidrug resistance-associated protein (MRP1) *(11)*, are normally expressed in hematopoietic lineages. Progenitor cells (CD34+) expressed Pgp *(12)* but not MRP *(11)*, and lymphocytes expressed both genes *(11,13)*. Recently, besides MRP1, at least four other homologs of these proteins (MRP2–MRP5) were identified *(14)*. MRP3 and MRP5 were overexpressed in only a few cell lines, and the RNA levels did not seem to correlate with resistance to either doxorubicin or cisplatin. MRP4 was not overexpressed in any of the cell lines that were analyzed, whereas MRP2 (or *cMOAT*) was substantially overexpressed in several cell lines, and its RNA levels correlated with cisplatin but not doxorubicin resistance in a subset of resistant cell lines (Fig. 1).

4.1. How to Measure MDR1 (Pgp) and MRP1 Expression

4.1.1. MDR1/Pgp

Numerous studies have investigated the expression of the *MDR1* gene in hematologic malignancies. The major problem with this published series is the absence of standardized methods for detection and quantification of *MDR1* expression in tumor specimens.

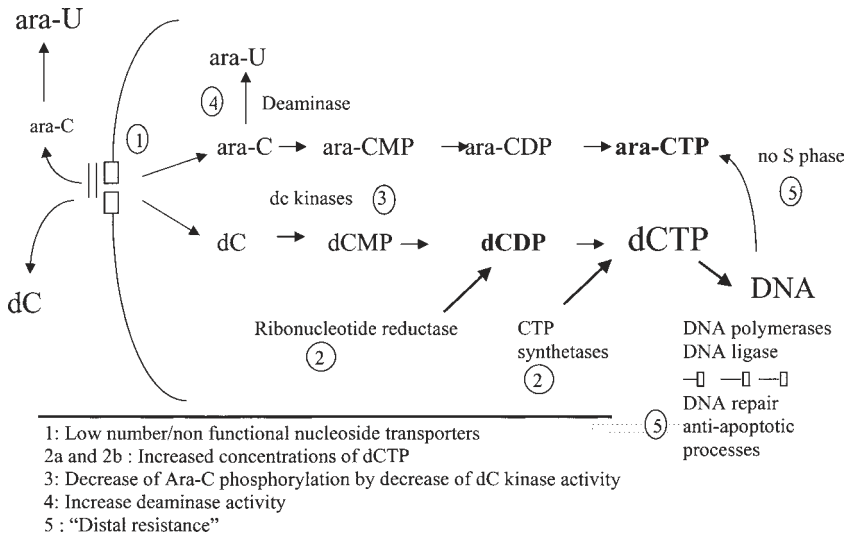

Fig. 2. Molecular basis for resistance to ara-C. Encircled numbers denote key steps in the resistance mechanism. dC, deoxycytidine. See text for forms of ara-C and dC kinase.

Several workshops *(15,16)* were held to develop a consensus on technical recommendations for measuring Pgp. The main recommendations are the following:

1. The tumor cell population in the sample must be as pure as possible, especially if "bulk" techniques are used [reverse transcriptase polymerase chain reaction (RT-PCR)].
2. Two different techniques should be used for validation, preferably with single-cell detection methods; protein detection and functional testing by flow cytometry are recommended for leukemic samples.
3. Calibration controls (all assays must be positive, including one cell line with a low level of positivity, comparable to clinical leukemic samples) and negative controls must be used. The same control cell lines have to be used by all centers working together.
4. A major confounding factor is the use of an arbitrary minimal cutoff for classifying samples as "positive" or "negative." It is recommended that the data be reported as continuous variables.

In addition, the most frequently used fluorochromes for clinical diagnosis [rhodamine [Rh]123 or calcein-AM] are generally not sensitive enough to detect the low-level resistant cells frequently isolated in clinical samples. Therefore, a novel sensitive functional assay may help us to gain a better understanding of the role of Pgp in AML. In a recent study we showed that JC-1, a fluorescent molecule with different emission wavelengths (green or red) according to its concentration, is a highly sensitive probe with which to assess Pgp activity in AML samples. As in cell lines *(17)*, the red emission band of JC-1 appeared to be more convenient for detection of low-level resistance in AML than did other probes, such as Rh123 or calcein-AM. With this probe we have defined an intermediate subgroup of patients, not detected by the Rh123 efflux assay, with an intermediate prognosis. With this assay, 65% of *de novo* AML patients expressed a functional Pgp molecule *(18)*.

4.1.2. MRP1

In contrast to reports on MDR1/Pgp, the published series of papers on MRP1 expression in AML present conflicting results regarding the predictive value of *MRP1* expression in drug resistance and overall survival *(19)*. Comparison of these results is difficult because of heterogenous patient populations and techniques for protein evaluation (RT-PCR, immunocytochemistry, flow cytometry, Western blot analysis, and so on), as well as differences in the definition of overexpression. In addition, the functionality of MRP1 was not assessed in most of the studies. Hence, as for MDR1/Pgp, it will be important to expand the consensus recommendations on evaluation of drug resistance to include MRP1.

In this regard, using a probe expelled by cells positive for both Pgp and MRP1, such as calcein-AM, one can simultaneously study the function of these two pumps *(20)*.

5. PHARMACOLOGIC RESISTANCE DUE TO ENZYME MODIFICATIONS

Resistance to antimetabolite drugs (e.g., methotrexate, hydroxyurea, fludarabine, and 2-chlorodeoxyadenosine) is mainly caused by intracellular enzymatic modifications *(21)*, even if transport is important *(22)*. Amplification of the dehydrofolate reductase gene after exposure to methotrexate is a well-known mechanism of resistance in many cell lines and is also found in clinical samples.

Another example of pharmacologic resistance is provided by the metabolism of cytarabine (ara-C), a nucleotide analog that plays a key role in the treatment of AML (Fig. 2). After transport into the cell through a nucleotide receptor ($hENT_1$), the first limiting step *(23)*, ara-C must be phosphorylated to its active triphosphate form (ara-CTP) by a series of kinase enzymes. The ability of fresh human leukemic cells to retain cellular concentrations of ara-CTP has been correlated with the duration of remission in patients treated with ara-C and an

anthracycline *(24)*. Although ara-CTP is an inhibitor of DNA polymerase A, it is also a substrate of this enzyme and is in competition with dCTP (the natural compound) for DNA incorporation. It is thought that the incorporation of ara-CTP into DNA is responsible for the lethal effect of this drug. The metabolism of ara-C is regulated by mechanisms similar to those that would normally control the formation of dCTP and depends on the activity of three enzymes: deoxycytidine kinase, 5'-nucleotidase (5NT), and DNA polymerase *(25)*. Mutations of ribonucleoside reductase (RNR) and CTP synthetase are frequent events in tumor cell models and confer resistance through the elevation of intranuclear dCTP pools.

6. DISTAL DRUG RESISTANCE

Excision and repair of lesions in DNA are caused by many specialized enzymes and are probably important for cell survival after exposure to genotoxic agents (e.g., anthracyclins, etoposide, alkylating agents, X-rays). Until now, no data have been available concerning the level of such enzymes in acute leukemia samples. The ultimate success of genotoxic anticancer agents is determined by the ability of malignant cells to initiate an apoptotic response to DNA damage *(26)*. Among the numerous factors known to modulate cancer-related apoptosis, p53 and the BCL2 family are the most extensively characterized proteins. p53 is activated in response to DNA damage and stops the cell in G1 phase (via p21). In case of nonfunctional p53, the threshold of DNA damage leading to apoptosis increases and could participate in drug resistance *(27)*.

Genetic alterations affecting $p16^{INK4a}$ and $p15^{INK4b}$, proteins that inhibit cdk-cyclin complexes and therefore the phosphorylation of the retinoblastoma protein (pRb) and also control exit from the G1 phase of the cell cycle *(28)*, are frequent in ALL and could be associated with a more aggressive course of the disease. The first evidence of in vitro correlation between pRb pathway alterations and cellular drug sensitivity was described in the past decade in solid tumors *(29)*, opening a new field of investigation for a better understanding of drug resistance.

The antiapoptotic properties of BCL2 and BCLXL, other members of the same family, are now well documented, and their overexpression has been shown to protect tumor cell lines from the toxicity of several chemotherapeutic agents *(30)*. Currently so many factors of the BCL2 family have been described (both pro- and antiapoptotic) that it is difficult to gain a comprehensive understanding of the pro- or antiapoptotic status of a cell by measuring only a few of the BCL2 members in clinical samples. This probably explains the contradictory results published in articles concerning these proteins.

In recent publications, Pgp has been postulated to act as a regulator of apoptosis *(31,32)*. The authors have shown that cells induced to express Pgp, either by drug selection or by retroviral gene transduction with *MDR1* cDNA, are resistant to cell death induced by a wide range of death stimuli that activate the caspase apoptotic cascade. However Pgp-expressing cells were not resistant to caspase-independent cell death mediated by pore-forming proteins and granzyme B. By this mechanism, Pgp could possibly have a role in resistance to ara-C in AML, but additional observations and experiments are required to support this hypothesis.

7. WHICH DRUG-RESISTANT MECHANISMS ARE IMPORTANT IN ACUTE LEUKEMIA?

7.1. Acute Myeloid Leukemia

7.1.1. Expression of MDR1/Pgp

The largest multicenter studies showed that from one-third to one-half of AML cases were Pgp-positive at diagnosis, whatever the detection technique used, with the proportion increasing with advancing age. The major impact on prognosis was on rates of induction treatment failure and overall survival, but not on disease-free survival, possibly owing to the high-dose chemotherapy given during consolidation (high-dose cytarabine or high-dose cyclophosphamide/total body irradiation). In monoparametric analysis, the MDR1 phenotype was as potent as "bad" cytogenetic features [e.g., 11q23, t(9;22), del 5,7 and complex abnormalities], age, or secondary leukemia. In multivariate analysis (Table 2), the MDR1 phenotype was an independent predictor of treatment failure, equaling or exceeding the power of cytogenetics and age in several studies.

This poor prognosis of the patients with AML expressing the MDR1 phenotype could be due either to a particular immature phenotype of leukemia or to the direct effect of the Pgp efflux pump on leukemic drug resistance to anthracyclines or etoposide, or both. The "mature" AML phenotype (CD33+, CD13+, CD117+, CD56+, and MPO+ expressed simultaneously) is associated with a very good prognosis and lower levels of functional Pgp, compared with AML lacking this feature *(33)*.

The immature CD34 phenotype is strongly linked with Pgp, as first emphasized by te Boekhorst et al. *(34)* and confirmed by all studies using sensitive antibodies against CD34. The function of Pgp is strictly correlated with expression of CD34, and the poor prognosis attributed to this phenotype could be caused by the active drug efflux by functional Pgp. In multiparametric analysis for prognostic factors including CD34 and Pgp expression, only Pgp appears to be significant. The expression of a functional Pgp molecule in leukemic cells with an immature phenotype contrasts most strikingly with the absence of Pgp in acute promyelocytic leukemia *(35,36)*, an observation confirmed by all studies to date.

Clinically, many studies have demonstrated the prognostic value of Pgp expression in AML. The resistance to anthracyclines and standard dose ara-C treatment is better correlated with Pgp than with global "treatment failure," including death during aplasia *(37)*. Interestingly, Nüssler et al. showed that the predictive potency of Pgp positivity observed in patients treated with classical 3+7 therapy was completely lost when the regimen included intermediate doses of ara-C *(38)*.

7.1.2. Expression of MRP1

Discordant results have also been published concerning the incidence of MRP1 expression in AML: the range of MRP expression is narrow compared with that of Pgp, and basal expression is found in all cases. A possible flaw in these studies is indicated by the high expression of this protein in normal mononuclear cells *(11)*, representing a risk of contamination of the sample, except when leukemic cells are gated by flow cytometry.

Table 2
Prognostic Role of MDR1 in Adult AML

			Probability of failure/resistance associated with MDR1 positivity[a]	
Author	*No. of patients*	*Evaluation of blast cells*	*Complete remission/resistance*	*Disease-free survival*
Campos et al. *(71)*	150	FCM (MRK16)	$p = 0.00001$	$p = 0.05$
Nüssler et al. *(38)*	166	FCM (C219/4E3)	$p = 0.002$	Not tested
Hunault et al. *(72)*	110	ARN/MRK16		
		Functional assay	Resistance, $p = 0.00001$	Not tested
vdHeuvel-E et al. *(73)*	130	ICC	$p = 0.01$	Not tested
del Poeta et al. *(74)*	158	FCM (C219 + JSB1)	$p = 0.001$	$p = 0.02$
Leith et al. *(37)*	211			
	(>65 yr)	FCM (MRK16)	$p = 0.004$	
		Functional assay	Resistance, $p = 0.0007$	Not significant
Willman et al. *(75)*	352			
	(<65 yr)	FCM (MRK16)	$p = 0.012$	
		Functional assay	Resistance, $p = 0.0007$	Not tested
Legrand et al. *(6)*	52	FCM (UIC2)		
		Functional assay	Resistance, $p = 0.03$	Not tested

Abbreviations: FCM, flow cytometry; DFS, disease-free survival; ICC, immunocytochemistry.
[a]All *p*-values from multivariate analysis.

Overall, 10–30% of patients presented with "high" MRP1 expression. MRP1 function, evaluated by the efflux of calcein, is not linked to CD34 expression, and a good correlation (better than for Pgp) has been observed between protein expression and dye efflux *(20)*.

7.1.3. Pgp/MRP1 Drug Efflux

Recently, we demonstrated that the best prediction of failure is obtained when dye efflux attributable to Pgp and MRP1 is measured at the same time *(6)*. Functional Pgp and MRP1 proteins are both able to decrease intracellular concentrations of anthracyclines and etoposide (but not cytarabine) within leukemic cells and are related to the in vitro and in vivo drug sensitivity. In our experience, using the MTT assay and fresh AML cells, Pgp and MRP1-related drug efflux measured by calcein-AM is highly correlated with daunorubicin in vitro cytotoxicity [inhibitory concentration of 50% (IC_{50})], and to a lesser extent with that of etoposide (VP16), but not cytarabine *(6)*.

7.1.4. Expression of LRP/MVP

The expression of lung resistance protein/major vault protein (LRP/MVP), a cytoplasmic non-ABC protein, was recently investigated with different techniques, and with different results. List at al. (39) and Pirker et al. (40), using immunocytochemistry, showed a strong correlation between LRP expression and treatment failure (39,40). On the other hand, Leith et al. (41), Michieli et al. (42), and Legrand et al. (43), using flow cytometry (and, for Legrand, RT-PCR), were unable to demonstrate any relation between LRP and treatment failure in the same category of patients. Using retrospective immunocytochemical study with LRP56 antibody applied to the same leukemic samples, we observed a relationship between LRP expression and lower overall survival *(6)*, raising the possibility of a threshold for positivity that is more easily observed with immunocytochemistry. Functional tests to measure LRP/MVP activity remain to be developed. Using the MTT assay to test in vitro drug resistance in fresh leukemic samples, we were unable to show any relationship between the expression of LRP and the IC_{50} of anthracyclines or etoposide *(6)*. We therefore concluded that, in adult AML, the major proteins involved in clinical resistance to the anthracycline are Pgp and MRP1, both of which are often associated with other factors predicting a poor prognosis.

7.1.5. Ara-C Resistance

dCMP deaminase activity plays a key role in resistance to ara-C in AML samples *(44)*. Using the MTT asay in 70 AML samples, we analyzed deoxycytidine kinase, dCMP deaminase, and in vitro resistance to ara-C. We have shown that samples with high levels of dCMP deaminase activity had a higher LC_{50} for ara-C and a poorer treatment outcome. These associations were not found with deoxycytidine kinase activity. Interestingly, the level of dCMP deaminase activity was correlated with the level of Pgp activity. In the same way, in vitro resistance to daunorubicin was correlated with in vitro resistance to ara-C *(45)*.

Using p21 expression as marker of genotoxicity, we demonstrated that high doses of ara-C (10^{-5} *M*), but not "standard" doses (10^{-6} *M*), are able to induce DNA damage in vitro in AML cells from clinical samples *(46)*.

7.2. Childhood AML

Only two studies of ABC proteins in children with AML *(47,48)*, using two consensus methods of analysis for Pgp measurement, have been published. Although the number of patients studied was low, the authors showed a global incidence

of MDR1/Pgp (+) lower than in adult AML (13–30%), and neither study showed a correlation between MDR1/Pgp and prognosis (complete remission, overall survival, disease-free survival). Nonetheless, Pearson et al. *(47)* described a close association between the expression of Pgp and t(8;21), a recognized prognostic factor in adult AML *(47)*. This association was not found in adult cases tested by the same authors. Thus, it seems that Pgp expression does not have the same predictive value in childhood AML as it does in adult AML.

7.3. Childhood Acute Lymphoblastic Leukemia

The main prognostic factor for long-term survival in ALL is sensitivity to corticosteroids, either in childhood *(5)* or in adults and elderly patients *(49)*. The cellular mechanisms of this resistance are not known. Although the number of glucocorticoid (GC) receptors could be involved *(50)*, the role of GC on repression of AP-1, leading to a decreased cell proliferation, was not confirmed in ALL *(51)*.

The incidence of *MDR1* overexpression in untreated patients with ALL was generally found to be low (<10%) at diagnosis and even at relapse, except during the most advanced stage of the disease, when clinical drug resistance is usually observed. The MDR1 phenotype was not predictive for induction treatment failure, whatever the method of detection used. This could be explained by the predominant role of corticosteroids in ALL therapy. Two studies, using flow cytometry and functional tests as recommended, found few positive cases at diagnosis and did not show any correlation between positivity and survival *(48,52)*. Fewer publications have concerned MRP in ALL, but all showed a measurable level of this protein at diagnosis, comparatively higher than in AML, and some cases showed an increase after treatment. The prognostic relevance of this expression has not yet been demonstrated *(48)*. Finally, den Boer et al. *(53)* found a correlation between LRP/MVP protein, but not Pgp or MRP1, and in vitro resistance to anthracyclines in childhood ALL.

In conclusion, clinical drug resistance in ALL is mainly the consequence of resistance to glucocorticoids, and efforts to understand the mechanisms of such resistance must be undertaken. The role of ABC proteins and LRP/MVP remains anecdotal, except perhaps after several relapses.

7.4. Adult Acute Lymphoblastic Leukemia

Apart from several studies in ALL in which some adult cases were mixed with childhood ALL cases, very few data are dedicated to adult ALL *(54,55)*, and none of them meet the criteria defined in the Memphis consensus. Wattel et al. *(55)*, using flow cytometry of JSB1 (an internal epitope), observed a high proportion of positive cases before treatment (32/50) but did not find any correlation between this expression and the complete remission rate, overall survival, or disease-free survival. In a small cohort of 23 adults tested by immunocytochemistry, Goasguen et al. *(54)* were the only authors to describe a correlation between Pgp expression (9/23) and failure to achieve complete remission. The absence of a large series tested with adequate methods precludes any conclusions concerning the prognostic significance of Pgp in adult ALL.

Deletion of $p15^{INK4b}$ and/or $p16^{INK4a}$, described in 20–30% of ALL cases *(56)*, was not associated with drug resistance in the latest publication concerning adult ALL *(57)*. Adult T-lymphoma-leukemia (ATL), which has a particularly bad prognosis, was investigated by immunoblotting by Kuwasaru et al. *(58)*, and found to be positive for Pgp in 8/20 cases at diagnosis, and in all 6 cases of relapse, refractory to polychemotherapy. The tax protein of human T-cell leukemia virus I (HTLVI) can promote MDR1 expression in cells transfected with the viral gene *(59)*, whereas MRP and LRP/MVP were found to be highly expressed in cases of ATL *(60)*.

8. HOW TO REVERSE DRUG RESISTANCE

8.1. Pgp Inhibition

8.1.1. Preclinical Studies

Numerous compounds have been identified that inhibit the efflux activity of Pgp and reverse cellular resistance in experimental systems (reviewed in ref. *61*). This led to the strategy of concomitant administration of chemotherapy and an MDR modulator to reverse clinical drug resistance. Modulators like verapamil and cyclosporine A also serve as substrates for Pgp, supporting the hypothesis of competitive ligands. Another way to inhibit Pgp efflux is to alter the phosphorylation pattern of the protein, through inhibition of protein kinase C. A precise delineation of Pgp drug-binding sites is necessary to elucidate the precise mechanisms of inhibition of each modulator.

The most advanced product designed to inhibit Pgp in clinical trials is the cyclosporin D analog PSC833 from Novartis, which was tested in phase III studies in multiple myeloma, in relapsed leukemia, and in elderly AML patients. PSC833 is 10-fold more potent than cyclosporin A and lacks any renal or immunosuppressive toxicity. The GlaxoWellcome compound GF120918 was also designed specifically to act on MDR modulation *(62)*.

8.1.2. Clinical Studies

The first large randomized study of MDR modulators in AML tested the usefulness of the addition of quinine to a combination of mitoxantrone and high doses of ara-C in 315 bad-prognosis adult leukemias (relapsing/refractory/secondary) *(63)*. Global results showed no difference between the quinine and control groups, but it was noted that (1) clinical drug resistance was higher in controls and (2) the toxic death rate was higher in the quinine group. The clinical toxicity of quinine could have masked the clinical benefit of MDR reversion. Final results of a randomized phase III study of daunorubicin and high doses of ara-C, with or without cyclosporine (16 mg/kg), in refractory/relapsing AML conducted by the Cancer and Leukemia Group B yielded encouraging results, with significantly higher disease-free and overall survival rates in patients receiving cyclosporine A with the anthracycline *(64)*.

Preliminary results of phase I trials of PSC833 showed the necessity of decreasing the dose of anthracycline and etoposide in elderly patients *(65)*. In two large multicenter studies in AML now under way, one in refractory/early relapsing disease treated with mitoxantrone, etoposide, and intermediate dose ara-C, and the other in untreated disease in elderly patients treated with daunorubicin and standard doses of ara-C, patients were randomized to receive PSC833 after stratification according to the Pgp status of each case.

An alternative treatment of relapsing AML consists of perfusion of an anti-CD33 antibody (CMA-676) linked with a potent antitumor antibiotic, calicheamicin. In a recent phase I study, a high rate of clinical response was observed only in MDR1-negative patients, characterized by low dye efflux in vitro *(66)*. This result could reflect the efflux of calicheamicin from resistant cells.

8.2. Modulation of MRP1

Drug efflux mediated by MRP1 is usually insensitive to classical Pgp-modifying agents but is sensitive to genistein, probenicid, MK-571, VX-170, and buthionin sulphoximine, a reduced glutathione (GSH) inhibitor that impedes the GSH-dependent transport of MRP substrates. Several modulators, such as probenicid *(20)*, a drug used to potentiate uric acid renal excretion, and VX-170 *(67)*, are candidates for clinical modulation of MRP.

8.3. Reversion of Other Types of Resistance

8.3.1. Resistance to Antimetabolites: the Ara-C Model

High-dose ara-C (1–3 g/m^2 every 12 h × 8–12) is a highly potent treatment for secondary/relapsing/refractory AML or ALL. Resistance to standard doses of ara-C (100–200 mg/m^2 per d × 7) is usually overcome by increasing the dose to offset the competition between this nucleoside and the natural deoxycytidine (dC): as more ara-C enters the cell, more will be phosphorylated and more will be incorporated into the DNA. The cellular concentration of ara-CTP in circulating leukemic cells during high dose infusions of the drug is highly correlated with clinical outcome *(68)*, confirming the importance of (1) ara-C uptake and (2) ara-C phosphorylation.

Another way to increase the ara-CTP DNA incorporation is to decrease the pool of dCTP. Hydroxyurea is a potent inhibitor of ribonucleotide reductase, the enzyme responsible for the conversion of CDT into dCDT. This enzyme could also be inhibited by high doses of thymidine. Clinical trials of treatments based on these pharmacologic interactions have been proposed *(69)*. It was also proposed to prime leukemic myeloid progenitors with growth factor [granulocyte or granylocyte/macrophage colony-stimulatioin factor (G-CSF, GM-CSF)] before ara-C treatment. After several large trials, the results of this approach remain inconclusive *(70)*.

8.3.2. Restoring Apoptosis

BCL2 overexpression could be inhibited by either preventing or disrupting the essential interaction between BCL2 and BAX, using small pharmacologic molecules or an antisense approach. Another possibility is to use GM-CSF and retinoids, based on the observation of downregulation of BCL2 in tumor cells exposed to differentiating agents or GM-CSF *(30)*.

9. CONCLUSIONS

Drug resistance in acute leukemia is multifactorial, and the mechanisms differ among disease subtypes. The mechanisms leading to a broad spectrum of resistance involve the ABC proteins and resistance to apoptosis via p53 or the BCL2 family. The roles of cell cycle regulatory proteins, namely, cyclins, cyclin inhibitors, and pocket proteins (Rb), are probably crucial in distal drug resistance and are beginning to be investigated.

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40 Drug Resistance in Acute Leukemia

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CONTENTS

1. INTRODUCTION

Today the cure rates in childhood acute lymphoblastic leukemia (ALL) approach nearly 80%, while the cure rate for acute myeloid leukemia (AML) continues to be between 35 and 50%, one of the lowest in all childhood cancers *(1)*. Drug resistance, either primary or acquired post-treatment, remains the main obstacle to improving the cure rate in AML. Studies of in vitro drug sensitivity show that, for many of the commonly used antineoplastic agents, AML blasts are relatively resistant compared to B-precursor ALL (BpALL) blasts *(2)*. For the most commonly used agents in AML, cytarabie (ara-C), anthracyclines, and etoposide, AML blast cells were either equally sensitive or 1.9- to 4.9-fold more resistant than BpALL blasts. In this chapter, we explore the possible bases for these observations.

Drug resistance can be inherent or can emerge after treatment. Specific drug resistance has emerged following the use of each of the drugs currently applied in childhood leukemias and is likely to emerge for new drugs as well, as is evident from the rapid development of resistance to STI-571 through upregulation of tyrosine kinase content *(3,4)*. For antimetabolites such as methotrexate (MTX) and ara-C, drug resistance is often mediated by multiple mechanisms including drug transport, the cellular content of target enzyme (protein) as well as alteration of the levels of various enzymes involved in determining the cellular concentration of the active metabolite of the drug *(5,6)*. With regard to chemotherapeutic agents belonging to the class of fungus-derived antibiotics or naturally occurring plant alkaloids, resistance appears to be derived largely from alterations in drug efflux mechanisms and to some extent changes in the levels of target proteins (e.g., topoisomerases) *(7,8)*. Resistance to alkylating agents is mediated through alterations in base excision repair pathways *(9)*. A generally puzzling but often noted phenomenon is that in in - vitro testing, blast cells are frequently sensitive or resistant to different classes of drugs, that is, resistance or sensitivity is often nonspecific, best exemplified by the experience in Down's syndrome AML blast cells *(10)* and in BpALL blasts *(11)*. Since most chemotherapeutic agents exert their cytotoxicity through the induction of apoptosis, it is likely that the cellular apoptotic regulatory elements play a key role in this regard *(12,13)*. Inasmuch as oxygen radical generation plays a key role in triggering and mediating apoptosis, the cellular redox status may be a major determinant of whether the cell is "primed" for apoptosis or not *(14)*.

Aside from these cell-specific mechanisms, drug sensitivity/resistance is also determined by host factors such as drug diffusion to sites of tumor, pharmacokinetics/pharmacodynamics, and mechanisms of drug detoxification *(15)*. Other factors not taken in to account in most discussions of in vivo drug resistance and sensitivity are the roles played by cytotoxic T-lymphocytes (CTLs), natural killer (NK) cells, and the clearing of partially damaged cells by the monocyte/macrophage system. These immunologic mechanisms may play a critical role in situations of low tumor burden. An unknown factor is whether cells resistant to induction of apoptosis by the mitochondrial pathway are also resistant to death signal or perforin/granzyme-mediated apoptotic pathways *(16)*. A discussion of these host factors is beyond the scope of this chapter.

2. METHODS FOR DRUG TESTING

Several methods are now available for in vitro testing of drug resistance/sensitivity.

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2.1. Cytotoxicity Assays

The most direct approach is to incubate the blast cell samples with the drug in question and determine cytotoxicity using a variety of methods, including the methyl-thiozol-tetrazolium (MTT) assay *(17,18)*, the differential staining cytotoxicity (DiSC) assay *(19)*, and cell survival by estimation of cell numbers before and after incubation. The advantage of these total cell kill assays is that they measure the end point of drug toxicity, cell kill. Of these, the most widely used test in childhood leukemias is the MTT assay developed by the Amsterdam group *(2,11,17,18)*. It takes advantage of the fact that viable cells reduce MTT to formazan and the change in optical density is proportional to the number of viable cells. Typically, the cells are incubated with varying concentrations of drug over 72–96 h in 96-well microplates, MTT is added at the end of incubation, and the change in optical density is measured colorimetrically, after which an IC_{50} value (drug concentration required for 50% cell kill) is derived. Limitations of the MTT assay are that it cannot be used for testing certain drugs (e.g., MTX); several experiments may be invaluable because of poor cell viability in the control wells; drug concentrations in the incubating mixture may decrease significantly over the time of incubation of 72–96 h (e.g., asparaginase); difficulty in correcting for an admixture with normal marrow cells; and the IC_{50} values may vary over several hundred fold. To overcome some of these obstacles, several apoptosis-based assays have been introduced: spontaneous cell survival in microcultures *(20)* and drug-induced apoptosis assays. The Wayne State group in Detroit has developed a semiquantitative drug-induced apoptosis (DIAP) assay that involves short incubations over 16 to 24 h *(21)*. This flow cytometry based-assay allows for recording events in individual cells and a more precise selection of the target population.

In the case of MTX, the nucleotides released by the dying cells appear to rescue other cells in patient samples over the duration of incubation and hence the MTT assay does not give valuable results *(22)*. To overcome this, Rots and the Amsterdam group adapted the thymidine synthase (TS) inhibition assay (TSIA), based on inhibition of the TS-catalyzed conversion of [^{3}H]dUMP (tritiated deoxyuridine monophosphate) to dTMP (deoxythymidine monophosphate) and ^3H_2O; this correlated with the MTT assay for antifolate sensitivity in four human leukemia cell lines with different modes of MTX resistance.

2.2. Drug Metabolism/ Surrogate Markers of Resistance

For many drugs, the correlation of clinical response with certain surrogate markers can also be used to derive resistance/sensitivity parameters (e.g., level of transport proteins, efflux proteins/functional efflux, drug retention, specific active metabolites, and target protein levels). Each of these parameters evaluates a particular aspect of drug metabolism. For example, MTX and ara-C are transported across the cell membrane by specific transporters, such as the reduced folate carrier 1 (RFC) *(22–24)* and nucleoside transporters, respectively *(25–28)*. .Each has been measured and correlated with drug transport and clinical drug sensitivity/resistance. For antibiotics and plant alkaloids, drug efflux has been shown to be the dominant mechanism of resistance in vitro. Drug efflux is largely mediated by transmembrane proteins containing an ATP-binding cassette (ABC) *(29–31)*. The most well-known and most functionally active ABC transporters are the P-glycoprotein (Pgp), a product of the multidrug resistance (*MDR1*) gene, the multidrug resistance-related protein (MRP), the lung cancer resistance protein (LRP, also called the major vault protein, MVP), and the breast cancer resistance protein (BCRP) *(32,33)*. A characteristic of these ABC transporters is that they transport multiple classes of drugs either intact or after conjugation with glutathione. Although antibiotics and alkaloids are the primary substrates for these ABC transporters, recent evidence suggests that MRPs may play a significant role in the efflux of MTX-GS-Hx conjugates *(34,35)*. The level of expression of these various efflux proteins can readily be measured by histochemistry, by fluorescent-tagged specific monoclonal antibodies or by measuring RNA using the reverse transcriptase-polymerase chain reaction (RT-PCR) *(31)*. *MDR1* and MRP are also susceptible to functional assay using the autofluorescence of anthracyclines and certain dyes, such as rhodamine 1,2,3 and calcein AM, with or without specific efflux blockers *(36)*.

Another productive approach for determining drug resistance/sensitivity is the measurement of the levels of active metabolites of drugs. This approach has been particularly useful in evaluating the effectiveness of antimetabolites, notably MTX, 6-mercaptopurine, and nucleoside analogs in the clinic. Because the levels of the active metabolites of the drugs are a function of the activity of the metabolizing and catabolizing enzymes, measurement of the activities of the enzymes or the respective gene transcript levels can also be used to derive resistance/sensitivity correlations. Measurement of target proteins is another surrogate marker for de novo or secondary resistance (e.g., dihydrofolate reductase in the case of MTX and levels of topoisomerases for anthracyclines).

2.3. Non-Specific Markers of Drug Resistance

Most drugs induce cell death by activation of the programmed cell death (apoptosis) pathway. The nuclear fragmentation and protein (DNA) degradation that precedes cell death is mediated by a cascade of proteases called caspases *(12,13)*. The whole process is regulated by a series of pro- and anti-apoptotic factors. The apoptosis machinery for deriving markers of drug resistance has been studied extensively. Oxygen radical generation is integral to this process and is involved in the initial cell protein/DNA injury, mitochondrial membrane depolarization and as well in the terminal injury to cell membrane. However, with regard to the oxygen radical metabolism, most attention has focused on glutathione (GSH) content and GSH transferases (GSTs), primarily as mediators of drug detoxification *(14,37–42)*. The clinical application of the results from these various methods will be discussed in the context of studies with selected drugs for ALL versus AML *(40,41)*.

3. DRUG RESISTANCE/SENSITIVITY PROFILES IN CHILDHOOD ALL

3.1. Studies with the MTT Assay

The most commonly used drugs in the treatment of childhood ALL are prednisone (Pred), dexamethasone (Dxm), vin-

cristine (VCR), Asp, MTX, 6-mercaptopurine (6-MP), doxorubicin, and ara-C. Drug resistance profiles for all of these agents except for MTX can be determined by the MTT assay popularized by Pieters and colleagues *(2,11,17,18)* and corroborated independently by Hongo et al.*(43)*. A brief summary of these studies follows.

There was generally no significant association between drug resistance and sex, leukemic cell burden, or FAB type *(44,45)*. Infants younger than 1.5 yr were significantly more resistant to Pred (>500-fold), Asp (11-fold), and VM26 (2.7-fold), but significantly more sensitive to ara-C (2.3-fold) than were ALL patients in the intermediate age group. When analyzing infants younger than 1 yr, similar results were found *(45)*. Pro-B ALL (CD10-negative B-precursor ALL) cells were significantly more resistant to glucocorticoids, Asp, thiopurines, anthracyclines and ifosfamide compared with common ALL/pre-B ALL but more sensitive to ara-C. Cells from children older than 10 yr were significantly more resistant to Pred, Dxm, Asp, idarubicin, and 6-MP. T-ALL cells showed a strong resistance to Pred, Asp and VCR and a mild but significant resistance to all other drugs except thiopurines and VM26. The favorable prognosis associated with DNA hyperdiploidy in childhood common ALL was linked to a relative sensitivity of hyperdiploid common ALL cells to antimetabolites, especially to 6-MP and ASP *(46)*. Kaspers et al.*(11)*, using at-risk group-stratified analyses, also showed that in vitro resistance to prednisolone, ASP, and VCR were each significantly related ($p < 0.01$) to the probability of disease-free survival (pDFS) after combination chemotherapy *(11)*. The combined data for prednisolone, ASP, and VCR provided a drug-resistance profile with prognostic independent significance that was superior to that of any single drug or any other factor. The 3-yr pDFS was 100% for the group with the most sensitive profile (20% of all patients), 84% (SE 6%) for the group with an intermediate sensitivity profile (40% of all patients), and 43% (SE 8%) for the group with the most resistant profile ($p < 0.001$). Similar predictive ability of sensitivity to two or more drugs was noted by Hongo et al.*(43)* from Japan, thus validating the observations of the Dutch group. At relapse, ALL blasts were found to be significantly more resistant to glucocorticoids, ASP, anthracyclines, and thiopurines, but not to vinca alkaloids, ara-C, ifosfamide, and the epipodophyllotoxins *(47)*.

Such in vitro studies have also been shown to predict for minimal residual disease (MRD) status at the end of induction *(48)*. MRD at d 15 and at end of induction (MRD-PI) correlated significantly with the in vitro resistance to prednisolone, but not to VCR or doxorubicin. The correlations between MRD and in vitro prednisolone resistance were even more pronounced when B-cell precursor and T-cell leukemias were analyzed separately. Interestingly, an earlier study from the same group had shown that in vitro resistance to prednisolone correlated strongly with resistance to 12 other drugs tested *(49)*. It is to be noted that the Berlin-Frankfurt-Münster (BFM) group in Germany had successfully used prednisone response as a basis for risk group stratification. Thus, in vitro sensitivity to individual drugs and MRD status at end of induction are apparently measuring the overall sensitivity of ALL blast cells to drugs and could be used to identify subgroups of patients requiring additional specific therapeutic strategies.

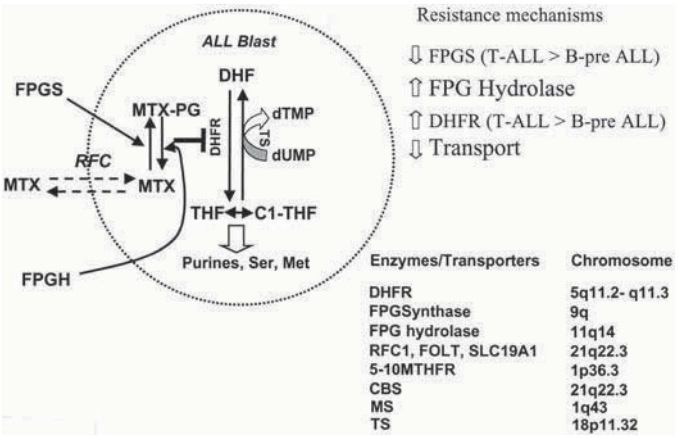

Fig. 1. Methotrexate: cellular uptake and mechanism of action. *Abbreviations*: MTX, methotrexate; MTX-PG, polyglutamylated MTX; RFC, reduced folate carrier; THF, Tetrahydrofolate; DHF, dihydrofolate; DHFR, DHF reductase; TS, thymidylate synthase; FPGS, folypolyglutamate synthase; FPGH, folypolyglutamate hydrolase; CBS, cystathionine beta synthase; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase; Ser, serine; Met, methionine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate.

These in vitro studies have also provided a possible basis for introducing specific therapeutic interventions in certain subgroups of ALL patients. Thus, infant leukemias were more sensitive to ara-C *(45)* and *TEL/AML1*+ blasts than to ASP, *(50)* suggesting that high-dose ara-C-based regimens may benefit infants with ALL and that intensive ASP may be useful in the *TEL/AML1*+ subgroup.

3.2. Mechanisms of Resistance/ Sensitivity in ALL

As informative as they are, studies with total cell kill assays do not provide information on the mechanistic basis for the differences between response and outcome observed in vitro as well as in vivo. Drug metabolism studies with individual drugs and use of surrogate markers have improved our understanding of some of the observed differences in outcome in various ALL subgroups. Since MTX and 6-MP form the mainstay of postremission strategies, several investigators have explored various parameters that may influence response to these agents. The complexity of studying each of the antimetabolites is illustrated by the various enzymes and transporters involved in their metabolism (Table 1).

3.2.1. Methotrexate Sensitivity/Resistance

Figure 1 illustrates some of the parameters tested with regard to MTX, an antifolate first introduced some 50 years ago in the therapy for childhood ALL. For a comprehensive review of the mechanisms of MTX sensitivity/resistance, the reader is referred to a recent review by Rots et al.*(5)*. The better outcome in hyperdiploid ALL has been linked to higher levels of polygluta-mylated MTX (MTX-PG) *(51)* and higher MTX transport based on the increased copy number of the chromosome 21-localized specific transporter, the *RFC* gene

Table 1
Some Critical Steps Involved in Antimetabolite Metabolism

Drug	*Enzymes/ transporters*	*Chromosome localization*
Methotrexate	DHFR	5q11.2-q11.3
	FPG synthase	9q
	FPG hydrolase	11q14
	MTHFR	1p36.3
	CBS	21q22.3
	MS	1q43
	TS	18p11.32
	RFC1, FOLT, SLC19A1	21q22.3
	MRP 1/2	16p13.1/10q24
6-Mercaptopurine	TPMT	6p11.3
	CBS	21q22.3
	MRP5	3q27
L-asparaginase	ASP synthetase	7q21-21
Cytarabine	dCk	4q13.3-q21.1
	dCD	1p36.2-p35
	CBS	21q22.3
	RR1/RR2	11p15.5/2p25-24
	Pyrimidine 5^1 nucleotidase I/II	7p15-14/7q23-25
	Thymidine kinase 2	16q22
	Nucleoside transporters	
	hENT1	16p21
	hCNTs	15q15-26

Abbreviations: See legends to Figs. 1 and 2.

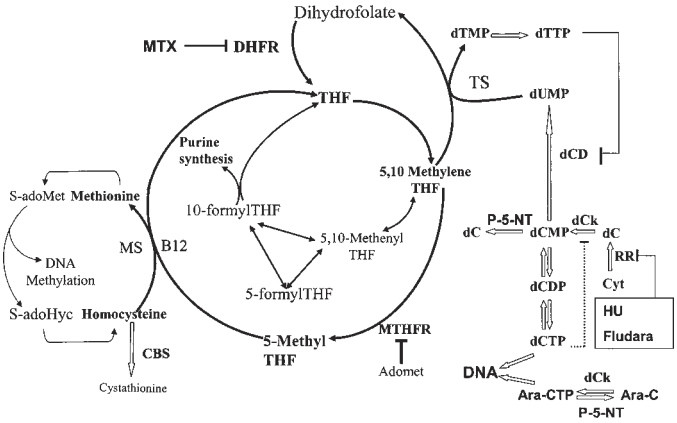

Fig. 2. Folate-B12 pathway. *Abbreviations:* CBS, MTHFR, purine synthesis, and dNTPs. S-adoMet, S-adenosyl methionine; S-adoHyc, S-adenosyl homocysteine; DTTP, deoxythymidine triphosphate; dCD, deoxycytidine deaminase; DCK, deoxycytidine urinase; P-S-NT, pyrimidine 5^1 nucleotidase; RR, ribonucleotide reductase; Cyt, cytidine; dC, deoxycytidine; DCMR, dC monophosphate; dCDP, dC diphosphate; dCTP, dC triphosphate;ara-C, cytarabine; ara-CTP, ara-C triphosphate; Hu, hydroxyurea; Fludara, fludarabine. Other abbreviations are defined in legend to Fig. 1.

(52,53). The relative resistance of T-ALL versus BpALL to standard-dose MTX regimens has been linked to an increase in the cellular content of dihydrofolate reductase (DHFR) *(54)* and low levels of a polyglutamylating enzyme, folypolygluatmylase (FPGS) *(55)*. Accordingly, the BFM group has shown improved survival with high dose MTX consolidation. Cellular DHFR content may also play a role in the previously observed, somewhat poorer outcome of ALL in African-American as compared to white Caucasian children, and in Hispanic children *(56, 57)*. Over time, with use of intermediate-dose and high-dose MTX, these differences have narrowed, suggesting a possible role for the use of high-dose MTX in consolidation therapy. Preliminary studies suggested that the cellular content of dihydrofolate reductase, the target enzyme for MTX, may be increased in African-American children *(57)*, providing a possible basis for the inferior outcome with standard-dose chemotherapy, and the improved outcome with the introduction of high-dose MTX *(58)*.

An aspect of MTX metabolism that has not been fully explored is the role of the commonly occurring reduced activity variant (C677T) of methylene tetrahydrofolate reductase (MTHFR). It is known that about 12% of North American and European populations may be homozygous and about 30% heterozygous for this variant *(59)*. The mutation results in reduced conversion of 5,10-methylene THF to 5-methyl THF, resulting in reduced cycling of homocystine to methionine (Fig. 2). Whether there is any specific effect on the end product of folate metabolism itself (i.e., thymidine synthesis) is unclear *(60)*. Nevertheless, the possibility that the mutation might alter the response to MTX cannot be excluded. In some studies, there was an association with increased systemic toxicity *(61,62)*. Prior clinical studies in childhood ALL suggested a correlation of transaminase elevation during maintenance with clinical outcome *(63)*. In a preliminary collaborative study, homozygous C677T patients exhibited greater sensitivity to MTX in the TSIA test *(64)*.

3.2.2. 6-Mercaptopurine and Polymorphisms in Thiopurine Methyltransferase

Thiopurine methyltransferase (TPMT) catalyzes the s-methylation of thiopurines, including 6-MP and 6-thioguanine. The TPMT activity exhibits genetic polymorphism with about 1 in 300 persons inheriting TPMT deficiency as an autosomal recessive trait *(65)*. When treated with standard-dose of thiopurines, TPMT-deficient patients may experience severe hematologic toxicity because of excessive accumulation of 6-thioguanine nucleotides (6-TGNs) and even death *(66)*. Red cell thioguanines have been shown to correlate with outcome in childhood ALL *(66,67)*.

To date, eight TPMT alleles have been identified, including three (TPMT *2, TPMT *3a, and TPMT *3c) which account for 80–95% of the intermediate or low enzyme activity cases.*(65)*. Population-based studies seem to suggest that low activity alleles are less common among Asian populations, ranging from 2% in South Asia to 4.7% in China, compared with a 10–14% incidence in African and European populations. This suggests that South Asian patients might require, on average, a somewhat higher dose of 6-MP to obtain the same therapeutic effect and same red cell 6-TGN concentrations. It is clear from these

data that antimetabolite sensitivity or resistance is a complex state influenced not only by leukemia-specific cellular events, but also by host-specific polymorphisms.

3.2.3. Effect of Polymorphisms of Cytochromes p450 and Glutathione S-transferases

Carcinogen-metabolizing enzymes are grouped into two categories: (1) phase I enzymes that modify functional groups on the drugs (oxidation/reduction), such as cytochromes p450 (CyP), alcohol dehydrogenase, and diaphorases, and (2) phase II enzymes (e.g., glucuronyl transferases and glutathionine S-transferases, or GSTs), which conjugate drug/toxin with endogenous substrates *(15)*. It has been proposed that polymorphisms in genes encoding for carcinogen-metabolizing enzymes may be relevant in determining susceptibility to cancer based on the presence or absence of either an active form of the enzymes involved in the activation of the carcinogen or an allele that is less efficient at detoxifying carcinogens.

Population-based studies suggested that the CyP1A1*2A allele is associated with an increased risk for ALL, whereas the CyP1A1A*4 allele may be protective *(68)*. Similarly, deletions of the phase II enzymes GSTT1 and GSTM1 are associated with an increased risk for ALL and AML, presumably due to deficient detoxification of carcinogenic metabolites from xenobiotics *(69)*. Interestingly, the polymorphisms in CyP1A1 also have an impact on the clinical outcome. Children with wild-type CyP1A1 had a better initial response and better event-free survival than those with the mutant type; homozygosity for the mutant phenotype was associated with an increased risk for death during induction and second malignancy *(68)*. Death during induction represents increased toxicity from some of the chemotherapeutic agents. The same group also noted that GSTT1 null type (absence of GST theta) or mutant GSTP(pi)1 was associated with a better response and better long-term survival than the GSTT1+ and wild-type GSTP1 groups. Since these polymorphisms are shared both by the somatic cells as well as the malignant clone, one conclusion might be that the absence of critical detoxifying enzymes might lead to increased systemic toxicity, rendering the leukemic cells more responsive to the agent, while resulting in a better leukemia-free survival. Investigators from the Children's Cancer Group have shown that the GSTM1 null type was significantly increased in childhood AML/MDS cases compared with controls, and there was a trend toward an increase in the GSTT1 null type as well. Furthermore, there appeared to be an increased frequency of GSTM1 null type in acute promyelocytic leukemia cases *(69)*. In preliminary studies, the GSTT null phenotype was associated with a reduced survival, predominantly due to an increased risk of death from toxicity in the more intensively treated patients *(70,71)*.

Thus, there is now sufficient evidence to suggest that genetic polymorphisms involved in the detoxification of xenobiotics play an important role in the causation of malignancies. Evidence is also emerging to show that such polymorphisms may have a significant impact on clinical outcome. The racial/ethnic distribution of the polymorphisms have not been fully elucidated *(72)*. It is important that as new protocol-based therapy is being developed, the potential impact of outcome based on polymorphic variations of drug-metabolizing enzymes be recognized.

4. DRUG RESISTANCE STUDIES IN AML

The most significant improvement in AML therapy occurred with the introduction of the anthracycline/ara-C drug combination for induction of remission *(73)* followed by intensification of therapy *(74)*. Subsequently, it was observed that resistance to conventional dose ara-C could be overcome by higher doses of ara-C *(75,76)*. The discovery of drug efflux, first by *MDR1(77,78)* and later the other ABC transporters [MRP *(79)* and BCRP *(32,33)*] and the major vault protein [MVP/LRP *(80,81)*], as a mechanism of in vitro resistance to anthracyclines led to intensive study of the clinical relevance of expression on leukemic blasts of the products of these ABC transporters. Furthermore, several studies using drug efflux reversal agents have been conducted with the hope that such strategies might improve current outcomes. On the other hand, empiric studies to date show that ara-C may be an equal if not more important component of the drugs used in AML. Randomized clinical studies in adults show that higher doses or longer durations of ara-C during induction and or consolidation improves outcome *(82–85)*. While no such large-scale studies have been done in children, a review of published studies suggest that the dose intensity of both ara-C and daunorubicin during induction is a significant factor in the improved results observed in the BFM-88/93 *(86,87)*, MRC-10/12 *(88,89)*, intensive timing CCG-2861 *(90)* studies over that achieved with the standard 3+7 dosing schedule in the POG-8821study *(91)* or the standard timing induction in CCG-2861 *(90)*. Despite these innovations in therapy, outcomes remain poor and the major cause of treatment failure is drug-resistant leukemia.

4.1. In Vitro Drug Resistance Studies

The limited success of AML therapy contrasts with that in childhood ALL. It is clear from clinical studies that AML is resistant to the standard drugs used in ALL, namely, prednisolone, VCR, Asp, MTX and 6-MP. In vitro studies confirm the observed clinical resistance to these drugs relative to ALL *(2)* Resistance of AML cells to VCR and to some extent other drugs appears to be linked to the expression of myeloperoxidase in AML blasts *(92,93)*. By contrast, recent studies have suggested that FAB M5 leukemia may be sensitive to ASP because of low levels of asparagine synthetase *(94)* and that some AML subtypes maybe sensitive to continuous infusion MTX *(95–97)*. Studies are under way in Europe in relapsed AML cases to test these new applications of old drugs in the treatment of AML.

Only a limited number of studies have prospectively evaluated in vitro drug resistance studies in AML with clinical outcome *(98–100)*. Early studies with the MTT assay in small groups of children with AML suggested that poor responders showed resistance to ara-C and that at relapse blast cells were three-fold more resistant to ara-C compared to initial diagnostic samples *(98)*. A subsequent study in adult AML patients suggested that resistance to ara-C, not to daunorubicin, was predictive of risk for relapse *(99)*. In a preliminary report of a large prospective study of childhood AML, POG investigators

showed that the best predictor for achieving remission was sensitivity to ara-C compared to sensitivity to daunorubicin or MDR1 expression *(100)*. Other studies in adults suggested a correlation of resistance to ara-C with age *(101, 102)*. In one study, the IC50 values for ara-C in pediatric AML cases were 178 ng/mL, 356 ng/mL in young adults, and 584 ng/mL in patients 60 yr of age or older *(101)*. In the same study, secondary AML cases exhibited greater resistance to ara-C than de novo AML cases. Since secondary AML is more common in adults than in children, *(103)* it is possible that some of these observed differences are linked to differing frequencies of subgroups of AML. As in ALL, evidence is emerging that sensitivity to two drugs may be a better predictor of outcome than sensitivity or resistance to one drug or other clinical/cytogenetic parameters *(104)*.

The Wayne State group used the MTT assay to provide in vitro evidence for the reported superior survival of AML children with Down's syndrome *(105,106)*. In brief, Taub et al. *(106)* were able to show that Down's syndrome AML blasts were more sensitive to ara-C and daunorubicin than blast cells from patients without this disorder. These observations have since been confirmed *(107)*. Thus, when used appropriately, in vitro cytotoxicity data can corroborate clinical observations and suggest novel uses of old drugs in the therapy of AML.

4.2. Mechanisms of Drug Resistance

4.2.1. Resistance to Ara-C and Nucleoside Analogs

The prototype drug ara-C is transported across the cell membrane by the nucleoside transporters (NTs) and then is phosphorylated to ara-C monophosphate, diphosphate, and the metabolically active triphosphate (ara-CTP) by deoxy-cytidine kinase (dCK) (Fig. 2) *(109,110)*. Phosphorylation appears to be the rate-limiting step and a common mechanism of resistance is downregulation of the activity of dCK *(110–112)*. Generation of ara-CTP can be inhibited by increased activity of deoxycytidine deaminase(dCD), prior to phosphorylation or by dephosphorylation of ara-CMP by 5'-nucleotidase *(113)*. Ara-CTP and other nucleoside analog (NA) triphosphates exert their effects by incorporation into DNA, resulting in chain termination and cell death, and by inhibiting RNA polymerases. An additional activity of some NAs is inhibition of ribonucleotide reductase (RR), which reduces the generation of deoxycytidine triphosphate (dCTP). Reduced dCTP may favor increased incorporation of NA-TPs into DNA.*(110)*.

There is strong clinical as well as in vitro evidence to suggest a direct relationship of ara-C cytotoxicity with ara-CTP generation *(114–118)*. The ability of higher doses of ara-C to overcome resistance to conventional doses supports these observations. However, ara-CTP generation in vitro can be saturated and excessive concentrations actually lower ara-CTP levels *(117 ,118)*. Thus, it is not clear what constitutes the optimal "high dose" of ara-C based on the in vitro kinetics of ara-CTP generation. From a clinical standpoint, drug diffusion into sanctuary sites, notably the central nervous system (CNS), has to be taken into account as well. Nevertheless, it is clear from clinical data that 6 to 12 doses of high-dose ara-C (3 g/m2/dose) during the induction and consolidation phases of therapy give the best results.

Because of the potential for multiple doses of ara-C at 3 g/m2 to cause severe morbidity, including cerebellar necrosis *(75)*, alternative approaches to modulate ara-CTP generation or lower dCTP levels have been developed. Fludarabine, an ara-C analog, enhances ara-CTP incorporation by inhibiting ribonucleotide reductase (Fig. 2), thereby lowering the levels of dCTP *(119–121)*. Hydroxyurea acts in a similar manner, but is a weaker inhibitor of ribonucleotide reductase *(121,122)*. MTX in appropriate doses can also augment ara-CTP generation *(123,124)* by lowering deoxythymidine triphosphate (dTTP) levels, and the subsequent release of inhibition of dCD by lowering dTTP levels; dCMP/dCTP levels fall and dCK is upregulated because of the lack of feedback inhibition by dCTP. The upregulated dCK favors ara-C phosphorylation. That these modulations can be achieved in the clinical setting have been shown with the FLAG regimen (fludarabine/ara-C/G-CSF) *(120)*, the hydroxyurea (HU)-ara-C combination *(121,122)*, and MTX/ara-C combinations *(123,142)*. Other attempts to upregulate dCK include the use of 5-azacytidine to cause hypomethylation of dCK and thereby its upregulation *(125)*. Although clinical data suggest that such modulation can overcome ara-C resistance, and despite the fact that promoter of dCK has GC regions *(126,127)*, it is not clear that dCK is regulated by promoter hypomethylation *(128)*. Studies in patients with Down's syndrome also show that the increased expression of dCK is not due to promoter hypomethylation, despite other evidence for global hypomethylation of genes in this disorder *(106)*. On the other hand, the increased sensitivity of Down's syndrome myeloblasts to ara-C has been shown to be associated with an increase in ara-CTP generation and low endogenous dCTP pools, presumably because of the increased activity of the chromosome 21-localized cystathionine-β-synthase and the resultant low dTTP pools *(129)*.

The above observations have led to an intense search for mechanisms of de novo resistance to ara-C in AML; the reader is referred to a recent review by Galmarini et al. *(110)* on this topic. Abnormalities of each component of nucleoside analog metabolism seem to play a role, individually or collectively, in resistant cell lines.

There appears to be some specificity of nucleoside transporters for each of the analogs. Thus, ara-C is predominantly transported by hENT1 (human equilibrative transporter 1), which is sensitive to inhibition by nitrobenzylmercaptopurine ribonucleoside (NBMPR) but not by hCNTs (human concentrative transporters), which can transport nucleosides across a concentration gradient; gemcitibine, on the other hand, is transported by both hENT1 and hCNT1 *(111)*. In clinical samples, resistance to ara-C has not yet been clearly linked to alterations in the density of NT sites, but some studies have shown that NT density may vary from 500 to 27,600/cell *(130)*. Furthermore, these studies showed that the lower sensitivity of T-ALL compared to AML blast cells correlated with a correspondingly lower number of NBMPR sites *(131)*. The availability of the fluorescent probe SAENTA for measuring NT sites might provide a means for testing for NT deficiency as a cause of clinical resistance to ara-C *(132,133)*.

The rate-regulating step in ara-CTP generation is catalyzed by dCK, whose activity is low in quiescent cells and increases

several fold during DNA synthesis. Low dCK activity has been shown in ara-C resistant cell lines; such cells are also frequently resistant to other nucleoside analogs. However, resistance is present only when the activity is reduced below 10% of normal *(111)*. Low dCK activity has been linked to ara-C resistance in some studies *(112,125)*. Possible causes for low activity in cell lines include hypermethylation of the promoter, inactivating mutations and deletions, or alternatively spliced isoforms. An early study of relapsed patients showed a high frequency of mutations in both coding and noncoding sequences of dCK *(134)*. However, a recent study of a large group of relapsed and refractory patients, using stringent methods, failed to corroborate this early study *(135)*. Other recent studies found alternatively spliced forms of dCK associated with low dCK activity in blasts from resistant but not from sensitive patients *(136)*. The experience in Down's syndrome suggests that posttranslational upregulation of dCK may enhance sensitivity to ara-C. It is not clear whether the opposite is a frequent mechanism of resistance to ara-C, although some earlier studies suggested hypermethylation of the promoter in some patients *(125)*.

Decreased generation of ara-CTP due to increased activity of cytosolic 5'-nucleotidase (cyto-5'-NU) and/or cytidine deaminase (dCD) have been studied in clinical samples with mixed results. Levels of cyto-5'-NU have been correlated with outcome in adult AML, chronic lymphocytic leukemia, and hairy cell leukemia *(137)*. Such studies are yet to be done in childhood AML. Similarly, studies showing both a positive correlation of outcome with dCD activity and a lack of correlation with outcome have been reported *(111)*. As in the case of dCK, structural alterations in dCD are a cause of in vitro resistance to ara-C in cell lines *(138)*. Such structural alterations, however, do not seem to play a major role in clinical drug resistance.

In summary, the effectiveness of ara-C depends on a multistep process of transport, phosphorylation, and catabolism. Posttranslational regulation of the activity of several key enzymes involved seems to play a major role in determining resistance or sensitivity. The sensitivity may also be linked to the general susceptibility of cells to apoptosis, as in the case of myeloblasts in Down's syndrome.

4.2.2. Resistance Mechanisms for Anthracyclines

Active outward transport of anthracyclines as mechanism of resistance was recognized in the early 1970s *(77)*. Subsequent work by Ling et al.*(77,139)*, linked this anthracycline efflux with the expression of a 170-bDa glycoprotein (Pgp), the product of the *MDR1* gene, now localized to chromosome 7q21.1. Since then, other members of the ABC (ATP binding cassette) transporter family have been discovered, and include the multidrug resistance-related protein (MRRP) *(79)*, the lung cancer resistance related protein (LRP, chromosome 16p13.2), now frequently referred to as the major vault protein (MVP) *(80)*, and the breast cancer resistance protein (BCRP, chromosome 4q22) *(32,33)*. MDR1 (Pgp), MRP1, and BCRP are expressed at the cell surface, while LRP is predominantly cytosolic. Substrates include bacterial and fungus-derived antibiotics (anthracyclines, actinomycin-D), plant alkaloids (vinca alkaloids, epipodophyllotoxins), semisynthetic and synthetic analogs, and some organic compounds (arsenoids, MTX) *(31)*. While these drugs differ in size and chemical composition, they share a general hydrophobic and amphipathic character, being usually lipid soluble and possessing a positive charge at neutral pH *(31)*. A study of the biochemical characteristics of drug transport via Pgp led to the identification of reversal of efflux by calcium channel blockers and several other diverse group of compounds including cyclosporine A and its analogs, *(31,140)* calmodulin inhibitors, *(141)* reserpine, *(142)* estrogen antagonists, and quinolones *(31)*. Inhibitors of MRP1 (e.g., probenecid, genistin, and indomethacin) *(31)* and of BCRP (fumitremorgin C) *(143)* have also been identified.

4.3. MDR1 and Drug Efflux — Clinical Relevance

It is now possible to quantify the expression of many of the drug transporters by flow cytometry using monoclonal antibodies. Furthermore, the discovery that many fluorochromes may be substrates for MDR1 led to the development of functional assays, both for MDR1 and MRP *(141–142)*. A plethora of studies looking at expression, functional efflux, and clinical correlations have been conducted, primarily in adults, to identify patients exhibiting resistance to anthracyclines and determine whether such resistance can be modulated by efflux blockers. Several of these adult studies have suggested that MDR1 expression is associated with a poor response and decreased survival, and that at relapse MDR1 is overexpressed *(7)*. Other studies have failed to show a lack of correlation. These results *(144–165)* are summarized in Tables 2 and 3.

An inherent problem in the early studies of MDR1 and drug efflux were the differences in the techniques used for measuring MDR1/MRP — immunohistochemistry vs PCR-based techniques *(31)*. The development of highly sensitive monoclonal antibodies have provided a more effective use of flow cytometric-based techniques, which have become the predominant method used in current evaluations. However, the lack of a consensus definition of overexpression continues to hinder comparative evaluation of these studies, namely, the percentage of cells expressing MDR1 *(146, 149)* vs MRK16 (or 4E3, UIC2) iso-ratio *(159)* or the derived statistic of Kolmogorov–Smirnov *(157,164)*. These determinations are generally compared against an in vitro functional assay or cytotoxicity assay to determine what level of expression constitutes true "overexpression." A common strategy is to include in the assay system control cells with known sensitivity and resistance profiles.

Even with rigorous standardization, in studies with simultaneous protein expression and functional assays on the same sample, there is not a perfect correlation between the two. In almost all studies, there are samples showing increased efflux on functional assays without overexpression of MDR1 — or for that matter — other ABC transporters. Additionally, differences result from comparison of single institution studies on freshly drawn samples with multicenter studies on samples. These theoretical and practical considerations with regard to measurement of MDR1 expression and drug efflux have previously been reviewed *(166–169)*. In a comprehensive study that relied on radioactive assays to measure anthracycline transport, flow cytometric and fluorescence microscopy for

Table 2
Prognostic Significance of MDR1 in de Novo AML[a]

					Correlation with outcome	
Author	*Citations*	*Year*	*No. of patients*	*a/c*	*CR*	*Survival*
Campos	146	1992	150	a	S	S
Zhou	147	1992	51	a	S	S
Marie	148	1993	42	a	S	–
Ino	149	1994	52	a	NS	NS
Wood	151	1994	54	a	S	NS
Del Poeta	150	1994	117	a	S	S
Sievers	153	1995	130	c	NS	NS
Schuurhuis	152	1995	17	a	NS	NS
List	154	1996	21	a	S	S
Goasguen	155	1996	25	a + c	NS	NS
van den Heuvel	156	1997	120	a + c	S	S
Willman	157	1997	349	a*	S	S
Willman	157	1997	203	a#	S	S
Leith	158	1997	352	a	S	S
Legrand	36	1998	53	a	S	–
Broxterman	159	2000	98	a	NS	NS

Abbreviations: a/c, adults/children; CR, complete remission; S, significant; NS, not significant; –, not evaluated; *, <55 years of age; #, >55 years of age.
[a]Adapted from doctoral thesis of Marrie van den Heuvel-Eberink, Erasmus University, Rotterdam, NL (personal communication).

Table 3
Prognostic Significance of MRP1, LRP and BCRP in de Novo AML[a]

					Correlation with outcome	
Author	*Citations*	*Year*	*No. of patients*	*a/c*	*CR*	*Survival*
MRP1						
Zhou	160	1995	52	a	NS	–
Te Boekhorst	161	1995	35	a	NSS	NS
Legrand	33	1998	53	a	NS(E)	–
						S(F) –
Borg	162	1998	91	a	NS	NS
LRP/MVP						
List	163	1996	21	a	S	S
Leith	164	1997	352	a	NS	NS
Willman	157	1997	349	a*	NS	NS
Willman	157	1997	203	a#	NS	NS
Legrand	36	1998	53	a	NS	–
BCRP						
Ross	165	2000	14	a	S?	–

Abbreviations: S, significant; NS, not significant; S?, trend toward significance in small patient sample; F, functional assay; E, expression level in small group of patients; a/c, adults/children; CR, complete remission; -, not evaluated; *, <55 years of age; #, >55 years of age.
[a]Adapted from doctoral thesis of Marrie van den Heuvel-Eberink, Erasmus University, Rotterdam, NL (personal communication)

protein expression, functional assays and comparison with in vitro MTT assay for daunorubicin, Broxterman et al.*(168)* showed that the effect of Pgp inhibitor on daunorubicin accumulation varies 30% (±), absolute daunorubicin accumulation varies 300% (±), and daunorubicin IC_{50} values in the MTT assay vary 1500% (±). Since it is not clearly established that the MTT assay for daunorubicin is predictive for treatment outcome, using daunorubicin IC_{50} values as a reference for reliability of other assays for Pgp may not be advisable. Comparisons with clinical outcome also may not be reliable inasmuch as most patients receive multiagent chemotherapy for remission induction, and thus a patient who is resistant to daunorubicin may achieve remission with ara-C or vice versa. Based on these observations, Broxterman et al.*(168)*. calculated that in AML blast samples expressing MRK16 at the level of the KB8 control cells (8 × MDR1 expression compared with sensitive KB-cells), daunorubicin accumulation might decrease to about 0.65 relative to Pgp-negative

samples. Since it is uncommon in clinical samples to see Pgp expression more than three times normal, it is not clear, with the current drug dosages, whether such modest reductions in accumulation are clinically relevant.

With regard to childhood AML, few studies have prospectively compared the relevance of expression of ABC transporters with outcome *(100,153,170,171)*. Preliminary results of the studies that have been completed to date show that Pgp expression in the pediatric age group is lower than what has been encountered in adult studies. It appears that Pgp expression in diagnostic blast cell samples increases with age. Thus, in pediatric patients, Pgp expression is detectable in about 15–17% of cases of de novo AML, *(100,153)* consistent with values in adults aged 20–35 yr compared to 27% for those between the ages of 35 and 49, 39% for those 50–65, and up to 70% in those over 65 yr *(158,164)*. Since MDR1 expression can be induced with exposure to xenobiotics including anthracyclines, as well as certain natural products such as grapefruit juice, *(172–174)* it is conceivable that with age, there is a nonspecific induction of MDR1 in certain individuals. These data suggest that the level of MDR1 expression may not be intrinsic to the leukemic cells (i.e., might reflect the MDR1 status in the somatic environment as a whole) and may not always be linked to a specific leukemic cytogenetic event. This is further illustrated by the observations in AML with monosomy 7/7q–, which is associated with poor prognosis and the contrasting superior outcome in AML with inv16. van den Heuvel-Eberink et al.*(175)* found no evidence for decreased expression or activity of MDR1 in monosomy 7/7q– cases, contrary to what one may have expected from the localization of MDR1 on 7q. A similar lack of loss of function of MRP in MRP1-deleted inv16 cases (MRP and LRP are localized to 16p31-32) has also been demonstrated *(176)*, suggesting that the good prognosis of inv16 may not be linked to decreased anthracycline efflux as originally proposed *(177)*. van den Heuvel-Eberink et al.*(178)* also looked critically at MDR1 expression in paired samples in the same patients from diagnosis and relapse, and found no evidence of increased expression/efflux or evidence for *MDR1* gene-related clonal selection at relapse.

Of considerable importance is the concept that MDR1 expression in AML may not necessarily have a negative implication. For example, MDR1 overexpressing cells exhibit increased collateral sensitivity to nucleoside analogs *(179,180)*. Supporting this finding are the clinical observations that MDR1 is overexpressed in AML1 with t(8;21) *(181)* and in AML blasts from Down's syndrome patients *(182)*. Both AML with t(8;21) and AML in Down's syndrome are highly sensitive to high-dose ara-C-containing regimens *(85,182)* and are associated with a superior outcome.

It is not surprising from the foregoing discussion that studies with the drug-reversal agents cyclosporine A or PSC 933 for improving treatment response and long-term outcome have produced mixed results *(183–186)*. In a Medical Research Council (MRC) trial, *(183)* the addition of cyclosporine during induction produced no significant difference in outcome. However, the negative results in the MRC study may in part have been due to a cyclosporine dose regimen inadequate for Pgp blockade. In the Southwest Oncology Group (SWOG) # 9126 trial, higher doses of cyclosporine (16 mg/kg/d), yielding a mean blood concentration exceeding 1600 ng/mL (a dose sufficient for modulation of Ppg-mediated efflux) produced a decrease in induction resistance and possibly increased relapse-free survival and overall survival *(184)*. Cyclosporine-treated patients had higher mean values for daunorubicin concentration. Among the treated patients, there was a correlation of response with d 9 daunorubicin concentration, but such a correlation was not noted in the group without cyclosporine treatment; thus, response rates were different in patients with comparable daunorubicin concentrations based on whether or not they have received cyclosporine. These data would suggest that the cyclosporine may have a non-Pgp linked beneficial effect on the response rate by increasing the cytotoxicity of daunorubicin *(184)*.

A preliminary review of the large prospective randomized trial of cyclosporine during consolidation by the Pediatric Oncology Group (POG 9421) also demonstrated a probable nonspecific benefit from this agent *(187)*. In the POG # 9421 study, the patients were randomized to receive standard-dose during induction versus high-dose ara-C during induction and postremission; both groups were randomized to receive drug combinations with or without cyclosporine. The control arm with standard 3+7 daunorubicin and ara-C and no cyclosporine had an inexplicably low remission induction rate. Nonetheless, a positive effect was noted when the two cyclosporine arms were compared with those not receiving this agent. Correlations with Pgp expression are not yet available from this study, but the low expression of Pgp in pediatric patients suggests that any benefit may not be all Pgp-linked. Cyclosporine can also block MRP- and BCRP-related drug efflux in a dose-dependent fashion. As suggested by List et al.*(184)* it is possible that some of the benefit is through the total blockade of efflux rather than the one mediated by MDR1 alone. Studies examining the expression and correlation of these other transporters with outcome of patients with AML are listed in Table 3. Alternatively, through its interaction with cyclophilin, cyclosporine may suppress angiogenic responses to vascular endothelial growth factor (VEGF), a cytokine implicated in myeloblast cell renewal and which confers an adverse prognosis in AML *(188)*. Another possibility is that cyclosporine may potentiate the cytotoxicity of several drugs through augmentation of ceramide-mediated apoptosis, by inhibiting glycosylation of ceramide via the glucoceramide synthase pathway *(189)*. Several MDR reversal agents appear to inhibit glucoceramide synthase, suggesting that their clinical effect may be in part related to this mechanism.

4.4. Non-Drug-Efflux-Related Resistance to Anthracyclines

The resistance/sensitivity mechanisms not related to ABC transporters include:

1. Alterations in the target enzyme topoisomerase II.
2. Metabolism of reactive oxygen species (ROS), especially the relationship to glutathione and glutathione S-transferases.
3. Pro- and anti-apoptotic machinery of the cell.

4.4.1. Topoisomerase II Levels and Resistance to Anthracyclines

The relationship of topoisomerase II (Topo II) function to drug resistance has recently been reviewed *(8,190–196)*. Topo

II, together with helicases, facilitates transcription and replication of DNA in the course of normal DNA synthesis. In the presence of Topo-II poisons like anthracyclines and epipodophyllotoxins, stabilization of the intermediary Topo-II-DNA covalent complexes result in a double-strand break that eventually culminates in apoptosis of the cell *(190)*. It follows that cells containing more Topo-II molecules will sustain more DNA damage and more cytotoxicity than cells containing fewer active Topo-II molecules. However, to date, there is no convincing evidence that a decrease in the Topo-II levels is a mechanism of resistance *(191,192)*. Studies of paired samples failed to identify a difference in the expression of Topo-II alpha at diagnosis or relapse *(193,194)*, nor is there evidence of a relationship of Topo-II alpha mRNA levels and sensitivity to daunorubicin or teniposide by MTT assay *(195)*.

4.4.2. Role of Reactive Oxygen Species

The generation of reactive oxygen species (ROS) and ROS-mediated cell injury is an important component of the activity of anthracyclines and several other drugs *(194, 197, 198)*. Drug-induced cytotoxicity is largely mediated through activation of the mitochondrial pathway of apoptosis *(12,13,197)*. In this process, ROS cause mitochondrial membrane depolarization and release of cytochrome P450 with subsequent triggering of the caspase cascade. The mitochondrial membrane depolarization can be measured by ROS-dependent reduction of fluorescence of $DiOC_6$. ROS generated by the drugs can also directly result in DNA damage as well as peroxidation of the membrane lipids. The cell is replete with redundant antioxidant machinery, including superoxide dismutase, glutathione, glutathione peroxidase, catalase, vitamin E and thioredoxins *(14,198–203)*.

In addition to direct detoxification of ROS, glutathionine may also confer resistance to anthracyclines and certain organic anions, such as MTX, by forming a drug-glutathionine complex with subsequent efflux through ABC transporters *(34.35)*. Because of the notion that ROS generation is a major component of the alkylating agent therapy, much early work focused on ROS in terms of depleting cellular GSH as a means of modulating drug resistance in solid tumors *(39–41)*. Two lines of investigations ensued: (1) the estimation of the GSH content itself and (2) the role of glutathionine S-transferase in the conjugation of GSH with the drug. Considerable interest also centered around the role of GSH in relation to detoxification of xenobiotics and the risk for cancer *(68)*. In an earlier section of the chapter, we alluded to this in relation to childhood ALL and AML. The interest in GST polymorphisms have centered around those that result in a reduction of the activity. Thus, while the GST null phenotype may increase the risk for carcinogenesis, on the one hand, through ROS damage of DNA and resultant mutations, it can also increase the cytotoxicity of chemotherapeutic agents by preventing degradation of the ROS generated by these agents. There is preliminary evidence to support the idea that the risk for leukemia relapse is decreased while there is an increased risk for systemic toxicity *(70,71)*.

The GSH content and its relation to outcome in childhood leukemias has been explored in only a small number of studies. In the most comprehensive study to date, Kearns et al. determined that the GSH content of the AML cells was higher than that of ALL blast cells and within ALL, T-lineage cells had a higher GSH content than B-precursor cells *(41)*. There was also evidence of an increased risk for relapse with high GSH content. That oxygen radical-mediated cell injury plays a significant part in drug sensitivity, is also supported by the observations in AML cells from patients with Down's syndrome *(106,182)*. Aside from a theoretically grounded increase in ara-CTP generation, Down's syndrome cells also exhibit an increased sensitivity to anthracyclines and other agents *(108)* that cannot be explained by gene dosage of enzymes directly involved in the metabolism of these drugs. ROS generation and ROS mediated increased cellular apoptosis are major components of Down's syndrome pathophysiology *(204–206)*. It is intriguing that increased superoxide dismutase (SOD), in excess of a gene dosage effect, is not protective in Down's syndrome cells. Ravindranath and Taub hypothesize that this relationship is possibly linked to increased generation of hydrogen peroxide in the presence of SOD and the lack of subsequent degradation of hydroxyl radicals *(182)*. Evidence that a modest increase in SOD may in fact be harmful has been shown in Down's syndrome cells *(202,203)* and in other systems *(207,208)*. Savasan et al.*(209)* explored the role of inhibition or scavenging oxygen radicals in relation to drug-induced cytotoxicity using an apoptosis-based assay. In preliminary studies, there was evidence that butylated hydroxyanisole, an oxygen radical scavenger, reduced drug-induced apoptosis in up to 75% of the samples studied. Evidence is also accumulating to show that ROS are important mediators of the therapeutic effect of arsenic trioxide in promyelocytic leukemia *(210)*. Thus, it is clear that the role of ROS and their modulation is fertile ground for study in the childhood acute leukemias.

4.4.3. Cell Growth and Apoptosis: Relationship to Drug Resistance

Linked to these systems of drug resistance are the multiple signaling pathways that regulate leukemia cell growth, survival, and apoptosis. A growing number of initiation points and downstream switches are being added to the cellular machinery regulating these critical processes *(211,212)*. Such molecular switches can be triggered from the cell membrane, such as is observed with the Fas death receptor or cytokine receptors and their mutations. For example, recent studies have mechanistically linked activating mutations in some cytokine receptors to chemotherapeutic resistance in AML *(213–218)*. Activating mutations of the c-Kit receptor have been shown to preferentially activate STAT3 and PI3-K pathways, leading to cytokine-independent survival and growth as well as resistance to chemotherapeutic agents and ionizing radiation *(211,214,215)*. Activating mutations of the Flt-3 receptor, characterized by an internal tandem duplication (Flt-3/ITD), are strongly correlated with poor outcome in both children and adults with AML *(214–220)*. The complete set of cellular pathways that such activating mutations stimulate are currently unknown, but should ultimately provide additional information concerning mechanisms of drug resistance. Furthermore, the development of small molecules with speci-

ficity for the mutated receptors holds particular promise for therapeutic intervention *(218,219,221,222)*.

Bcl-2 and Bcl-XL have been shown to represent critical inhibitory regulators of apoptosis that function through the release of mitochondrial cytochrome c and caspase activation *(220,221,223–225)*. The upregulation of expression or activation of Bcl-2 and Bcl-XL has been associated with increased resistance in leukemia *(222–227)*. These apoptosis regulators have also served as potential targets for treatment. For example, using antisense oligonucleotides directed against Bcl-2, Keith et al.*(225,228)* demonstrated that a significant number of AML patient samples showed decreased Bcl-2 expression and increased apoptosis when exposed to ara-C *(225,228)*. The use of antisense oligonucleotides to Bcl-2 are currently in clinical trials in adults and children with AML *(226,229)*.

Mutations in p53, a critical regulator of cell cycle checkpoints and DNA repair, have been observed in AML, although a strict correlation with outcome has not been demonstrated. However, the function of p53 in drug resistance has been nicely modeled with the use of p53 deficient (p53 –/–) mouse embryonic fibroblasts transformed by adenovirus E1A protein *(227,230)*. This model has shown that p53 –/– fibroblasts were resistant to both ionizing radiation and several chemotherapeutic agents, while wild-type or heterozygotic fibroblast derived tumors showed increased sensitivity and apoptosis to such agents *(228,231)*. Because p53 mutations are common in a significant percentage of AML *(229,232)*, as well as other types of cancer, this pathway has been the focus of several different therapeutic approaches designed to reinstate normal p53 checkpoints. For example, some groups have introduced wild-type p53 into p53–/– tumor cell lines using adenovirus, demonstrating that increased apoptosis and growth inhibition results *(230,233)*. Other studies have shown that the introduction of wild-type p53 via an adenoviral vector into nude mice following subcutaneous implantation of p53–/– tumor cells prevented the establishment of the tumor cells *(231,232,234,235)*. A more "pharmacological" type of approach has been the development of "adapter" drugs or molecules that are able to restore the normal p53 structural configuration and function of mutant p53 *(233,236)*.

5. CONCLUSIONS

The primary goal of defining the drug resistance of leukemic cells is that a more complete understanding of this property will lead to more effective alternative therapies. While tremendous advances have been achieved in the identification of methods to measure leukemic cell drug resistance, there is still no consensus as to the optimal predictive method. The definition of in vitro resistance is also only one part of the edifice of drug resistance, with host factors, including both pharmacologic and pharmacogenetic characteristics, clearly playing an important role.

A desired consequence of defining resistance patterns is that specific molecular mechanisms of resistance can be identified and subsequently exploited therapeutically. While the identification of specific pathways can often be achieved, effectively targeting them has proven to be particularly challenging. For example, one issue has to do with the specificity and selectivity of the intervention. This issue is nicely demonstrated by clinical trials designed to inhibit specific drug efflux transporters, such as the MDR1 Pgp *(234,235,237,238)*. An additional problem is that rapid development of alternative pathways of resistance by tumor cells, as exemplified in the resistance mechanisms that arise in response to exposure of CML cells to STI-571, a tyrosine kinase inhibitor, directed to the BCR-ABL fusion protein. The emergence of amplified BCR-ABL genes and BCR-ABL mutations that alter drug/target interactions represent timely examples *(236,237,239,240)*. Importantly, the development of resistance to the molecularly targeted drug STI-571 is reminiscent of the types of resistance mechanisms observed with conventional chemotherapeutic agents *(235,237,238,241)*.

One hope is that combinations of multiple agents targeting specific pathways of growth, survival and resistance will result in improved outcomes for patients with leukemia. This strategy has certainly proven successful with the relatively few conventional chemotherapeutic regimens that have been developed and tested over the past several decades. However, most outcomes for patients with leukemia and other types of cancer have reached plateaus with these so-called conventional approaches. Whether combinations of conventional and molecularly targeted agents will result in significantly improved outcomes remains to be determined. This is a battle with an enemy that has proven to be quite resourceful, but like Medusa, may be defeated with a blade such as that wielded by Perseus. Let us hope that the two Gorgon and immortal sisters of Medusa do not have sufficient speed or cunning to anticipate our next therapeutic strategies.

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DARIO CAMPANA

CONTENTS

1. INTRODUCTION

In patients with acute leukemia, the effects of treatment on leukemic cells and normal hematopoietic cells are periodically monitored by examining the cellular composition of bone marrow samples. However, conventional microscopic techniques are limited in the sensitivity and accuracy with which they can estimate the extent of leukemic cytoreduction. In most cases, these methods cannot detect the presence of leukemic cells with certainty if they represent <5% of the bone marrow cell population. Therefore, in patients considered to be in remission by morphologic criteria, it is estimated that the hematopoietic tissue may still conceal 1×10^{10} malignant cells *(1)*. Because of this high threshold of detection, conventional morphologic studies cannot measure fluctuations in the leukemic tumor burden with any degree of accuracy, and recurrent disease can be diagnosed only when it becomes clinically overt.

Methods that allow the sensitive detection of residual leukemic cells have many potential applications in the clinical management of patients with acute leukemia, in addition to monitoring the response to chemotherapy and detecting impending clinical relapse. These methods can also potentially improve autologous stem cell transplantation by detecting residual leukemic cells in harvested bone marrow or peripheral blood and by assessing the efficacy of "purging" procedures. Measurements of residual disease could also serve as surrogate end points to allow comparison of the rates of leukemic cytoreduction achieved by different cytotoxic regimens and to determine the efficacy of novel antileukemic therapies.

The essential premises of minimal (submicroscopic) residual disease (MRD) studies are (1) that patients who are in clinical remission have different levels of submicroscopic leukemia and (2) that the level of residual disease measured in a sample of bone marrow provides a reliable estimate of the response to treatment and the likelihood of relapse. The assumption that leukemic cells are homogeneously distributed throughout the bone marrow implies that measurements of MRD performed on a small aliquot (equivalent to perhaps 0.1% of the total volume of active marrow) are representative of the total tumor burden. However, clinical observations *(2)* and results of experiments with animal models of MRD *(3)* indicate instead that there may be considerable heterogeneity in the distribution of leukemic cells after treatment. Another assumption is that the residual leukemic cells detected in MRD studies are viable cells that retain the capacity for self-renewal. In contrast to MRD methods that examine nucleic acid material [e.g., polymerase chain reaction (PCR)], those based on intact cells (e.g., flow cytometry) can determine the viability of cells. In principle, the self-renewal capacity of the cells can be assessed by sorting residual leukemic cells and inoculating them into a milieu that supports their growth *(4)*. In practice, such experiments are far too complex to perform on a routine basis. Therefore, the validity of these assumptions can be tested only indirectly, by correlating MRD findings with the outcome of treatment.

2. METHODS OF MRD DETECTION

2.1. Historical Notes

The first attempts to identify submicroscopic disease in patients with leukemia were made soon after the first antibodies for leukocyte differentiation antigens became available. The strong expression of the common acute lymphoblastic

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leukemia (ALL) antigen (CD10) and of terminal deoxynucleotidyl transferase (TdT) in leukemic cells and the virtual absence of cells expressing these markers in the peripheral blood suggested that these molecules could be used as markers of leukemia. However, it soon became apparent that a proportion of cells in the bone marrow (later identified as B-cell progenitors) expressed both CD10 and TdT *(5,6)*. TdT+/CD10+ cells are particularly abundant in the bone marrow of young children and in bone marrow regenerating after chemotherapy or transplantation, where they may represent 10–20% of the mononucleated cells *(7)*. Although TdT and CD10 may be abnormally expressed in some leukemic cases, these markers cannot be used alone to identify leukemic cells in the bone marrow. TdT+/CD10+ cells are extremely rare in peripheral blood, and early studies suggested that a proportion of TdT+ cells > 0.1% in this site could be used as an early sign of relapse *(8)*. However, subsequent studies showed that the proportion of TdT+ cells in peripheral blood often rises in patients who remain in remission *(9)*. CD10 as a single marker has also proved unsuitable for reliable MRD surveillance in peripheral blood *(10)*.

These early studies revealed that TdT+ cells in bone marrow do not express T-cell markers, whereas TdT+ T-lineage leukemic lymphoblasts (like normal thymocytes) do express them *(11,12)*. This immunophenotypic combination provided the first tool for the productive study of MRD in patients with acute leukemia *(13)*.

Over the next two decades, many methods of MRD study were tested, but few approaches proved to be sufficiently reliable *(14)*. For ALL, these included flow cytometric profiling of aberrant immunophenotypes, PCR amplification of fusion transcripts, and PCR amplification of antigen-receptor genes. Only the first two of these can be applied to acute myeloid leukemia (AML), because antigen-receptor gene rearrangements are not found in most cases of AML. Conventional karyotyping and fluorescence *in situ* hybridization are occasionally useful for clarifying the nature of morphologically ambiguous blast cells, but they cannot reliably detect submicroscopic leukemia *(15,16)*. Only a few laboratories have had success with methods based on differential properties of normal and leukemic cells in culture *(17,18)*. Uncertainties remain about these assays' reproducibility (based on potentially inconsistent factors such as fetal calf serum and leukocyte conditioning medium), sensitivity (the plating efficiency of leukemic cells is very low), and objectivity (visual identification of colonies is required).

Although they are already clinically informative, current methods of MRD detection are far from perfect, and efforts to improve their accuracy and reliability are ongoing. By its nature, detection of MRD presents unique technical challenges that go beyond those of the individual method used. Therefore, investigators who have technical expertise but little experience in MRD detection should exercise prudence by following tested methodologic protocols or by extensively testing newly developed approaches. At the same time, experienced investigators of MRD must meet the challenge of simplifying methods while maintaining or increasing their reliability, if these laboratory assays are to be used for maximal clinical benefit.

2.2. Flow Cytometry

Current strategies for flow cytometric detection of MRD rely on combinations of leukocyte markers that do not normally occur in cells of the peripheral blood and bone marrow. We identify such leukemia-associated phenotypes by quadruple-color immunologic staining techniques *(19)*. Triple-color flow cytometry may be sufficient in many cases, but an additional marker usually improves the discrimination of normal and leukemic cells. Newly developed instruments that allow simultaneous detection of five or more fluorochromes and the parallel development of antibodies conjugated to fluorochromes detectable by ultraviolet excitation should further improve the ability to discriminate between normal and leukemic cells. We include in our panels only marker combinations that allow the detection of one leukemic cell among 1×10^4 or more normal cells.

Differences in the antigenic expression of leukemic cells and normal progenitor cells may be qualitative, quantitative, or both *(19–23)*. Qualitative differences are seen when immunophenotypic combinations are expressed by leukemic cells but extremely rarely or never by normal bone marrow cells. Examples are the combinations CD34/CD19/CD21, which is expressed in a proportion of B-lineage ALL cases, and CD34/CD56, which is expressed in some cases of AML. Normal cells with these phenotypes are encountered on rare occasions, but antigen expression is usually well below that of leukemic blast cells. The expression of markers such as CD19, CD10, and CD34 in some cases of B-lineage ALL can be more than 10-fold that in normal B-cell progenitors (E. Coustan-Smith and D. Campana, unpublished data). Therefore, this overexpression can be used to distinguish leukemic blast cells from subsets of normal cells with similar phenotypes. Underexpression of CD45 and CD38 is also an abnormal feature in some B-lineage ALL cases (E. Coustan-Smith and D. Campana, unpublished data). Detection of MRD by flow cytometry in T-ALL relies on a different principle. In general, T-ALL cells express phenotypes that are characteristic of immature T-cells. However, because immature T-cells are normally confined to the thymus, bone marrow cells that express this phenotype are indicative of MRD in patients with T-ALL *(13,19,24)*. A detailed list of the markers used for MRD detection in our laboratory is presented in Table 1.

To monitor residual disease, it is essential to have detailed information about the immunophenotypic features of the patient's leukemic cells at the time of diagnosis. These dictate selection of the appropriate markers. If the immunophenotypes are not known, the full range of potentially useful markers has to be used; this expensive and time-consuming option might still fail to identify residual disease.

The proportion of cases that can currently be monitored for MRD by flow cytometry varies from laboratory to laboratory. Factors that determine this proportion include the number of markers tested, the use of bone marrow regenerating after chemotherapy to define the normal range of measurements, and the stringency with which the laboratory defines leukemia-associated immunophenotypes. Using immunophenotypes that allow us to detect one leukemic cell in 1×10^4 or more normal cells, we can currently study MRD in approx 90% of cases of ALL and 75% of cases of AML *(19)*.

Flow cytometric detection of MRD could be improved by the development of new leukemia markers that would increase the number of patients for whom MRD studies can be performed. In addition, because current panels for MRD detection are rather complex, a smaller number of markers applicable to a greater number of patients could increase the efficiency of testing and facilitate the establishment of flow cytometric MRD studies in other centers. To date, leukemia-specific phenotypes have been identified by systematically comparing the immunophenotypes of leukemic and normal bone marrow cells *(19)*. However, this process is slow, is largely based on trial and error, and is limited by the relatively small number of markers used for routine leukemia immunophenotyping. We have pioneered the use of cDNA arrays to identify immunophenotypic differences between ALL cells and normal lymphoid progenitors and have identified over 200 genes that are overexpressed in ALL and warrant further study *(25)*. The overexpression of some of these genes was confirmed by testing with specific antibodies *(25)*.

It is important to note that the identification of immunophenotypic differences between normal and leukemic cells in diagnostic samples is only a starting point. The expression of the selected markers by normal bone marrow cells must then be tested under different conditions. It is especially crucial to determine whether levels of expression remain consistent in bone marrow cells of patients undergoing chemotherapy, experiencing bone marrow regeneration after remission induction chemotherapy, or after hematopoietic cell transplantation. In addition, experiments in which leukemic cells are diluted with normal cells are required to test the sensitivity afforded by the new immunophenotypic combination. Finally, it is important to test the degree to which expression of the selected immunophenotypes fluctuates in response to chemotherapy. Samples that are collected early during treatment and contain clearly identifiable populations of leukemic blasts are useful for this purpose.

Table 1
Immunophenotypic Markers Used to Study Minimal Residual Disease (MRD) in Children with ALL

ALL subclass	*Expression difference between normal and leukemic cells*	*Phenotype*	*Frequency (%)*[a]
B-lineage	Quantitative[b]	CD19/CD34/CD10/TdT	30–50
		CD19/CD34/CD10/CD22	20–30
		CD19/CD34/CD10/CD38	30–50
		CD19/CD34/CD10/CD45	30–50
	Qualitative[c]	CD19/CD34/CD10/**CD13**	10–20
		CD19/CD34/CD10/**CD15**	5–10
		CD19/CD34/CD10/**CD33**	5–10
		CD19/CD34/CD10/**CD65**	5–10
		CD19/CD34/CD10/**CD21**	5–10
		CD19/CD34/CD10/**CD56**	5–10
		CD19/CD34/CD10/**CD66c**	10–20
		CD19/CD34/TdT/**cytoplasmic** μ	10–20
		CD19/CD34/TdT/**CD58**	30–50
		CD19/**7.1**	3–5
		CD19/**p53**	3–5
T-lineage	Ectopic[d]	TdT/CD3	90–95
		CD34/CD3	30–50

[a] Proportion of childhood ALL cases in which one leukemic cell in 1×10^4 normal bone marrow cells can be detected with the listed immunophenotypic combination. Most cases express more than one combination suitable for MRD studies *(19)*.

[b] Markers expressed with different intensity by normal and leukemic immature B-cells.

[c] Aberrant immunophenotypes found on leukemic cells but not normal cells. The aberrant marker is indicated in bold face type.

[d] Markers not expressed on bone marrow or peripheral blood cells but found on normal thymocytes.

2.3. PCR Amplification of Antigen-Receptor Genes

The greatest obstacle to the routine use of PCR amplification for MRD assays in ALL is the necessity of identifying immunoglobulin or T-cell receptor (TCR) gene rearrangements in each patient's malignant cells at diagnosis. The junction-specific nucleotides of these rearrangements are used to make oligonucleotides that serve as primers in the PCR procedure or are used as post-PCR hybridization probes to detect specifically the rearrangements of the malignant clone *(14,26)*. In many modern cancer centers, this procedure has been simplified by automation. Alternatively, genes can be amplified by using consensus primers *(27–29)* and the results then analyzed by gene scanning *(30)*. The sensitivity of this approach, however, is less than that afforded by patient-specific primers and is usually not greater than 1 in 10^3 cells.

There has been some debate about the optimal method for quantifying MRD. Dot-blot methods involve hybridization and detection of the junction-region oligonucleotide after PCR amplification *(31)*. MRD is quantified by comparing the test sample with similarly amplified, serially diluted DNA obtained at the time of diagnosis. Because of limited diversity in some rearrangements, the sensitivity of detection afforded by hybridization methods can range from 1×10^{-6} to 1×10^{-3}. Limiting-dilution PCR is based on detection of a single leukemic gene rearrangement by using two rounds of PCR amplification *(32–34)*. At the sample dilution at which both positive and negative results occur, the percentage of leukemic cells can be estimated by using Poisson statistics. Limiting-dilution PCR is relatively rapid (5–6 h, vs 1– 2 d for dot-blot procedures), has a uniform sensitivity, and provides quantitative assessment of MRD. The main drawback to this method is its cumbersome nature; a relatively large number of dilution replicates are required for sample analysis.

Real-time quantitative PCR (RQ-PCR) technology has great potential for MRD assessment *(35)*. This method allows detection of PCR products while amplification occurs, thus ensuring quantification of samples during the exponential phase of PCR. In addition, it eliminates post-PCR processing of samples. Although the synthesis of junction-specific fluorogenic probes is relatively expensive, this cost could be reduced with time and through alternative PCR strategies, such as those based on a limited number of consensus probes, positioned at the 3' end of V-regions or J-regions. Preclinical studies using RQ-PCR to amplify antigen-receptor genes with the ultimate aim of studying MRD in ALL have yielded encouraging results *(36,37)*.

IG and *TCR* genes can be analyzed successfully in only about 75–90% of ALL cases—the proportion of cases that have sufficiently leukemia-specific sequences *(26,31,38,39)*. Oligoclonal *IG* and *TCR* rearrangements, which are reported to occur in as many as 30% of cases (approx 15% at our institution; G.A. Neale, unpublished data) also pose problems in identifying reliable sequences for MRD monitoring *(40,41)*. However, the likelihood of false-negative results can be reduced if more than one leukemic rearrangement is monitored for each patient.

2.4. MRD Measurements by Detection of Fusion Transcripts

PCR amplification of DNA fusion sequences can be used for MRD studies only when the chromosomal breakpoints in different cases cluster in a relatively small area (e.g., <2 kb); for example, deletions in the *SIL/SCL* region in T-cell ALL create useful sequences of this type *(14)*. However, because the breakpoints of most recurrent translocations cover larger regions, fusion-gene mRNA molecules are used as reverse transcriptase (RT)-PCR targets instead. Examples in ALL include *BCR-ABL* transcripts in cases with the t(9;22), *E2A-PBX1* mRNA in cases with the t(1;19), *MLL-AF4* and *MLL-AF9* transcripts in cases with the t(4;11), and *TEL-AML1*. For AML, *AML1-ETO* is used in cases with the t(8;21), *PML-RARA* in cases with t(15;17), and *CBFB-MYH11* in cases with inv(16) *(42)*.

RT-PCR with nested primers can consistently detect one leukemic cell among 1×10^4 or more normal cells *(42)*. The stability of breakpoint-generated fusions during the disease course makes them useful tumor-specific PCR targets for MRD detection, although fewer than half of the cases of acute leukemia have specific chromosomal aberrations with well-defined breakpoint-fusion regions. Quantification of PCR products has been significantly improved by the development of RQ-PCR technology *(35)*. However, precise quantification of PCR products and conversion of the result to an accurate estimate of residual tumor burden remains challenging. The susceptibility of RNA to degradation may adversely affect MRD estimates, particularly in samples that are not processed immediately after collection. In addition, the relationship between the number of transcripts and number of residual tumor cells may be difficult to establish, because it is not yet known whether levels of transcripts fluctuate during therapy.

3. METHODOLOGIC CONTROVERSIES

Sensitivity is a crucial issue in evaluating methods of MRD detection. Clinical studies suggest that patients whose level of MRD is less than one leukemic cell in 1×10^4 have a particularly good prognosis *(38,43–45)*. Therefore, MRD methods should have a sensitivity of at least 1×10^{-4}. This level of sensitivity can be routinely achieved by using flow cytometry and a wide range of immunophenotypic combinations *(19)* and by PCR amplification of antigen-receptor genes, if patient-specific probes or primers are used *(31)*. Attempts to simplify these methods, for example, by reducing the number of markers used in flow cytometry or by avoiding the use of patient-specific oligonucleotides in PCR reactions, invariably decrease the sensitivity of the assays. However, the results of clinical correlative studies suggest that a 1×10^{-3} level of sensitivity may be sufficient to identify patients at a higher risk of relapse *(38,43–45)*. The level of sensitivity needed is largely determined by the clinical question to which the MRD assay is applied. For example, a sensitivity of 1×10^{-3} might yield sufficiently informative results during early stages of chemotherapy, but a higher level of sensitivity would be needed for the study of leukemic-cell contamination of stem cell autografts.

It has become apparent that quantitation of MRD, rather than its mere detection, is important in anticipating the relapse of leukemia. Whereas flow cytometry is naturally suited to the accurate quantitation of residual leukemic cells, RT-PCR amplification of chromosomal breakpoints may be more problematic. Several approaches have been proposed to improve MRD quantitation by PCR amplification of antigen-receptor genes *(31–33,46)*. In our experience, the use of multiple dilutions and Poisson analysis provides accurate estimates of MRD by this method *(39)*.

Highly sensitive methods should clearly be able to distinguish patients who have MRD levels $\geq 1 \times 10^{-4}$ from those who have lower levels of MRD, because the clinical outcomes of these two groups are different. Obviously, MRD assays should also recognize high levels of MRD (e.g., $> 1 \times 10^{-2}$), which predict a markedly high risk of relapse *(44,45)*. However, the usefulness of precise MRD quantification among patients with intermediate levels of detectable MRD has not been entirely established. If MRD measurement is used as a surrogate end point to measure the efficacy of treatment, then accurate quantitation across a wide range of values may be crucial.

How do various methods of MRD detection compare with each other? At our institution, MRD studies are performed by flow cytometry, by PCR amplification of *IGH* genes, and by RT-PCR amplification of fusion transcripts. To determine the correlation between measurements obtained by flow cytometry and PCR amplification of *IGH* genes, we assayed serial dilutions of normal and leukemic cells by both methods *(39)*. We found the two methods to be highly sensitive (1×10^{-4} or greater sensitivity), accurate (r^2 was 0.999 for flow cytometry and 0.960 for PCR by regression analysis), and concordant (r^2 = 0.962). We then used both methods to examine 62 bone marrow samples collected from children with ALL in clinical remission *(39)*. In 12 samples, both techniques detected MRD levels $\geq 1 \times 10^{-4}$. The percentages of leukemic cells measured by the two methods were highly correlated ($r^2 = 0.978$). Of the remaining 50 samples, 48 had MRD levels $< 1 \times 10^{-4}$. Results were discordant in only two of these samples: PCR detected two in 1×10^4 and five in 1×10^4 leukemic cells, whereas the results of the flow cytometric assays were negative; both patients remained in remission by clinical, flow cytometric, and molecular criteria, at 18 and 28 mo afterward.

We also compared the results of flow cytometry with those of RT-PCR amplification of fusion transcripts (*BCR-ABL* and *MLL-AF4*; E. Coustan-Smith, S. Shurtleff, D. Campana, J. Downing, et al., unpublished results). In 25 of 27 bone marrow samples collected during remission, the methods gave concordant results (10 were MRD+, and 15 were MRD–). Of the two remaining samples, one was negative by flow cytometry but positive (1×10^{-5}) by PCR; the other was positive by flow cytometry but negative by PCR (MRD was detectable by both

methods in prior and subsequent samples from this patient). These results indicate that measurements of MRD by our flow cytometric method and by PCR assay are comparable and that clinically significant levels of MRD (i.e., ≥ 1×10^{-4}) can be detected by either technique.

Specimens collected for MRD testing must often be shipped. Because of their complexity, tests used for MRD detection can currently be performed only in a few specialized laboratories. Although DNA sequences remain stable over time and may be detectable by PCR amplification even after cells have undergone apoptosis, RNA integrity may be seriously affected by delays in testing. Because most leukemic cells enter the apoptotic pathway as soon as they are removed from the in vivo microenvironment *(47)* and because this process may affect protein expression, flow cytometric studies may also yield imprecise results if samples are not tested immediately after collection. The shipment of cryopreserved cells, if feasible, might overcome this limitation. A more practical possibility is treatment of samples with a fixative or a stabilizer before shipment. Current investigations in our laboratory are aimed at optimizing such procedures. Because of the relatively wide availability of flow cytometry, detection of MRD by this technique is also amenable to decentralization. Recently developed methods that allow the rapid exchange of flow cytometric files between centers could be invaluable in ensuring the consistency of MRD measurements performed at separate centers *(48)*. The full realization of this objective would require the simplification of current flow cytometric panels.

Another controversial issue is the potential use of peripheral blood instead of bone marrow for MRD studies. Practical and ethical considerations limit the acquisition of sequential bone marrow samples from children. The procedure causes considerable discomfort, and even if performed under deep sedation, it requires extensive staff support and prolonged postsedation surveillance. Studies of the relative distribution of residual leukemic cells in the bone marrow and peripheral blood of children treated for ALL have so far yielded discordant results. For example, Brisco et al. *(49)*, using PCR amplification of *IGH* genes to quantify MRD in 35 paired blood and bone marrow samples from 15 children receiving induction therapy, found that although the level of MRD in peripheral blood was proportional to that in marrow, it was approx 10-fold lower. Conversely, Van Rhee et al. *(50)*, relying on RT-PCR amplification to study p190 *BCR-ABL* mRNA in 29 paired samples from 18 patients during treatment for Philadelphia chromosome-positive ALL, found no significant difference between the numbers of *BCR-ABL* transcripts detected in most sample pairs. In four pairs, the marrow was positive and blood was negative; however, RNA integrity was suboptimal in two of these sample pairs. In a further three pairs, more than 10 times as many *BCR-ABL* transcripts were found in the marrow than in the blood. Using the same approach, Martin et al. *(51)* studied nine paired samples from six patients and found that MRD levels in marrow exceeded those in blood by a factor of 10 or more in every case. More recently, Donovan et al. *(52)* used PCR amplification of antigen-receptor genes to compare MRD in 801 paired blood and bone marrow samples obtained from 165 patients: findings in 82% of the pairs were concordant.

We studied 718 pairs of bone marrow and peripheral blood samples. MRD was detected in both marrow and blood in 72 pairs and in marrow but not in blood in 67 pairs; it was undetectable in the remaining 579 pairs. Remarkably, findings in marrow and blood were completely concordant in the 150 paired samples from patients with T-lineage ALL: for each of the 35 positive marrow samples, the corresponding blood sample was positive. In B-lineage ALL, however, only 37 of the 104 positive marrow samples had a corresponding positive blood sample *(53)*.

Taken together, the available data suggest that the correlation between levels of MRD in the peripheral blood and bone marrow may vary with the time of measurement, the subtype of ALL, and possibly the type of treatment.

4. CLINICAL APPLICATION OF MRD STUDIES

Several studies have shown that MRD detected during clinical remission of ALL is independently associated with the outcome of treatment *(34,38,43–45,54–58)*. Therefore, the use of MRD assays to guide therapy promises to increase cure rates further by preventing overtreatment of patients with highly responsive disease and undertreatment of those with more aggressive leukemia. Although already clinically informative, current MRD assays are not perfect, and their optimal clinical use has not yet been established.

In two prospective multicenter studies that used PCR amplification of *IG* and *TCR* genes to monitor MRD in children with ALL *(38,45)*, the presence and quantity of MRD were significantly correlated with the risk of early relapse at each of the time points studied. In both studies, a high proportion (≥ 1×10^{-2}) of leukemic cells was associated with a higher rate of relapse. Rapid cytoreduction (i.e., low MRD levels after 15 d of treatment) is associated with an excellent outcome *(59)*. The potential clinical value of flow cytometric MRD studies was demonstrated by a prospective study of MRD in children with ALL treated at our institution *(43,44)*. The flow cytometric finding of MRD at any point during clinical remission was highly associated with subsequent clinical relapse.

In contrast to the results obtained by flow cytometry and PCR amplification of antigen-receptor genes, clinical outcome was not correlated with MRD measured by RT-PCR amplification of *E2A-PBX1* fusion transcripts at the end of consolidation treatment in one study of children with B-lineage ALL *(60)*. The disparity between this and other studies cannot be readily explained. Possible contributing factors include the different patient cohort studied (the RT-PCR study included only children with pre-B ALL and *E2A-PBX1* gene fusion), different therapy, and possible imprecise estimates of MRD caused by degradation of RNA during sample shipping. Notably, another study based on RT-PCR amplification of *MLL-AF4* transcripts found that PCR findings and treatment outcome were well correlated in 22 adults and 3 infants with ALL and t(4;11) *(58)*.

An intriguing report by Roberts et al. *(61)* described the use of quantitative PCR to measure MRD in 24 children with ALL in first remission. Seventeen patients remained in remission 2–35 mo after the completion of treatment. Fifteen of these patients showed evidence of residual leukemia, which was corroborated in seven patients by a colony assay for ALL cells.

The authors concluded that molecular signs of residual leukemia can persist for as long as 35 mo in children who have completed chemotherapy for ALL and are in remission; these findings suggest that eradication of all leukemic cells may not be a prerequisite for cure. Other studies have failed to confirm the high frequency of detectable leukemic cells or leukemia-specific PCR products in children with ALL who enjoy prolonged complete remission *(34,38,43,45)*. Thus, it is not known whether these findings might have been peculiar to a specific treatment protocol or might have been confounded by normal lymphoid cell clones with *IG* gene sequences similar to those of leukemic cells *(62)*. Although the colony assays corroborated the results of the PCR study in some samples, the specificity of the culture system used to support the growth of leukemic cells has been questioned *(62)*.

MRD measurements are also informative in the context of stem cell transplantation for patients with ALL. MRD monitoring of *BCR-ABL* fusion transcripts predicted the outcome of allogeneic or autologous bone marrow transplantation in patients with ALL and t(9;22) *(63)*. Knechtli et al. *(64)* showed that MRD detection by PCR amplification of antigen-receptor genes was predictive of subsequent relapse in children with ALL who underwent allogeneic bone marrow transplantation. The 2-yr event-free survival estimate was 0% for the 12 patients who had high levels of MRD (from 1×10^{-3} to 1×10^{-2}) before transplantation, 36% for the 11 who had low levels (1×10^{-3} to 1×10^{-5}), and 73% for the 33 who had undetectable MRD ($p < 0.001$). Not unexpectedly, the same group also found that the detection of MRD after transplantation is predictive of an unfavorable outcome *(65)*.

In acute promyelocytic leukemia, the PCR detection of *PML-RARA* transcripts indicates a high risk of relapse, and the eradication of these transcripts is now regarded as a goal of clinical management of this disease *(66,67)*. The significance of *AML1-ETO* mRNA in AML cases with t(8;21) is less clear. Early studies showed that this transcript can persist in the bone marrow or even the peripheral blood for as long as 12 yr of remission after the completion of treatment *(68–70)*. An explanation for this finding is provided by recent work of Miyamoto et al. *(71)*, who found *AML1-ETO* transcripts in a fraction of stem cells, monocytes, and B-cells in remission marrow and in a fraction of B-cells in t(8;21) leukemic marrow. These authors also demonstrated *AML1-ETO* transcripts in colony-forming cells of erythroid, granulocyte-macrophage, and/or megakaryocyte lineages in both leukemic and remission marrow. These studies suggest that this genetic abnormality originally occurs at the level of stem cells capable of differentiating into B-cells and myeloid lineages; a fraction of the *AML1-ETO*-expressing stem cells undergo additional oncogenic event(s), ultimately leading to transformation into AML. Despite the lack of strict association between *AML1-ETO* transcripts and AML cells, it was recently reported that careful quantitation of these transcripts can identify patients who are at risk of relapse *(72–74)*.

A few large studies of detection of residual AML by flow cytometry have been reported. Sievers et al. *(75)* found that the presence of MRD was correlated with earlier relapse of AML in 19 of 35 children in first morphologic remission. San Miguel et al. *(22)* performed sequential studies of MRD in 53 patients with AML. The level of MRD at the end of the induction and intensification phases of therapy was correlated with the probability of subsequent relapse. Patients who had MRD $> 5 \times 10^{-3}$ during first remission had a significantly higher rate of relapse (67%) than did patients who had a lesser level of MRD (20%; $p = 0.002$). Patients who had MRD $> 2 \times 10^{-3}$ at the end of intensification therapy had a relapse rate of 69%, whereas others had a relapse rate of only 32% ($p = 0.02$). In a study of 56 patients, Venditti et al. *(76)* found that an MRD level of 3.5×10^{-4} cells or higher after consolidation was significantly correlated with short duration of overall survival and short duration of relapse-free survival. Among patients who received autologous bone marrow transplantation for AML, the levels of cells expressing an aberrant immunophenotype in the autograft was correlated with disease recurrence *(77)*, highlighting the necessity of good purging techniques in autograft procedures *(78)*.

5. CONTROVERSIES IN THE CLINICAL APPLICATION OF MRD ASSAYS

The findings cited above strongly suggest that MRD studies should be incorporated into treatment protocols. Single measurements at informative time points during therapy may be sufficient in most cases. For example, the 5-yr cumulative incidence of relapse among patients with B-lineage ALL who do not have MRD at the end of remission induction is <10% *(79)*. Among patients who have MRD at this time point, a higher level of MRD ($\geq 1 \times 10^{-2}$) is strongly associated with subsequent relapse. It has been argued that children with ALL who have ultrarapid responses (no MRD after 2 wk of therapy) and who remain free of MRD may currently be overtreated. However, the excellent outcomes observed among MRD-negative patients were achieved through the use of fairly intensive therapies; therefore, reduction of treatment intensity or duration would not be without risk.

Despite the value of single measurements of MRD, however, sequential measurements are likely to provide additional information. In our series, for example, children with ALL whose MRD disappeared by wk 14 of continuation therapy had a cumulative incidence of relapse similar to that of patients who had no MRD at earlier time points *(44)*. By contrast, the persistence of MRD during continuation chemotherapy was increasingly associated with subsequent relapse. Thus, patients who have positive MRD findings at the end of remission induction therapy may benefit from more frequent MRD testing during clinical remission.

Although it would clearly be desirable to extend the potential benefits of MRD monitoring to all patients, there is no single technique that can be used for all patients. Therefore, multiple methods must be used to allow universal monitoring of MRD. At St. Jude, the simultaneous use of flow cytometry and PCR amplification of antigen-receptor genes has allowed us to study all of 237 consecutive cases of childhood ALL. This approach should also prevent false-negative results caused by changes in immunophenotype or predominant antigen-receptor gene clone during the course of the disease.

MRD studies also allow investigators to define the kinetics of response to antileukemic therapy with a level of accuracy unattainable until now. It remains to be decided how MRD

assays should be used to guide treatment. Should the results of MRD assays override traditional prognostic features? Because MRD assays measure the actual response to therapy, whereas prognostic features only *predict* the response to therapy, it would be logical to give more weight to MRD findings. However, oncologists may be reluctant to abandon clinical and biologic parameters, such as age, leukocyte count, and genetic features, whose relation to treatment response has been repeatedly confirmed even across different treatment protocols, until more clinical studies of MRD are completed. Therefore, at present, the prudent course would be to combine MRD with clinical and biologic parameters for the comprehensive risk assessment of patients with acute leukemia.

The use of MRD studies may improve the treatment of patients with acute leukemia in ways other than risk assessment. For example, the utility of autologous transplantation might be improved by combining effective techniques for purging the graft of leukemic cells with sensitive methods for detection of residual leukemic cells. In addition, MRD measurements may serve as surrogate end points for the clinical testing of novel therapeutic approaches, such as kinase inhibitors, cytokines, immunotoxins, adoptive T-cells, compounds that interfere with oncogenic molecular aberrations, and inhibitors of angiogenic growth factors, which may not be amenable to methods traditionally used for testing anticancer treatments.

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42 Molecular Monitoring of Minimal Residual Disease

GIOVANNI CAZZANIGA AND ANDREA BIONDI

CONTENTS

1. INTRODUCTION

Continuous clinical remission (CCR) in patients with acute leukemia, either lymphoid (ALL) or myeloid (AML), is conventionally defined to be <5% of bone marrow blasts by morphologic assessment. However, this relatively liberal description can be associated with a leukemic burden of up to 1×10^{10} blasts *(1)*. As shown in Fig. 1, the term *minimal residual disease* (MRD) has been used to define the lowest level of disease detectable in patients in CCR by the methods available. A number of techniques have been developed over the past 5–10 yr to complement morphology in assessing response to treatment, including immunologic, molecular, fluorescence *in situ* hybridization (FISH) and colony assays *(2)*. Despite notable progress with these methods, their sensitivities vary considerably, and several critical issues must be resolved before MRD determinations can be routinely considered in clinical decision making.

2. MOLECULAR MONITORING OF MRD

In recent years the genes involved in the most frequent chromosomal translocations have been identified, providing important insights into disease pathogenesis and normal cellular physiology *(3,4)*. Molecular assays for leukemic cells have been developed (reviewed in refs. *2*, *5* , and *6*) and now permit more accurate diagnosis of ALL and AML subtypes with frequently recurring translocations. DNA or cDNA obtained from RNA after a reverse-transcription (RT) step can be used in these assays, when there is a suitable molecular target. The polymerase chain reaction (PCR) assay is approx 400–4000 times more sensitive than Southern blot analysis *(7)*. In experiments in which the assay sensitivity was assessed by mixing leukemic and normal DNA or cells, the presence of a very small number of abnormal cells, in the range of 1 in 10^5 or 10^6, has been consistently detected *(8)*. Molecular assays have been applied to the identification of two types of "clone-specific" targets in ALL and AML: breakpoint fusion regions arising from chromosomal translocations and patient-specific sequences reflecting unique recombinations of antigen receptor genes.

3. MOLECULAR TARGETS FOR MRD ASSESSMENT

3.1. Tumor-Specific Translocations

Molecular characterization of clonal chromosomal abnormalities in blast cells from leukemia patients has had a profound impact on the understanding of the molecular changes involved in leukemogenesis *(2,9)*. Somatically acquired chromosomal translocations or inversions have been found in up to 65% of the acute leukemias. These structural rearrangements can have two main consequences. As with the translocations listed in Table 1, a protooncogene can be activated when the promoter and enhancer elements of distinct genes are juxtapositioned within the same locus *(2,9)*. Alternatively, discrete segments of two different genes may be joined as a result of the translocation, creating a fusion gene encoding a chimeric protein. The products of the latter aberrant genes are most often transcription factors or tyrosine kinases *(2,9)*.

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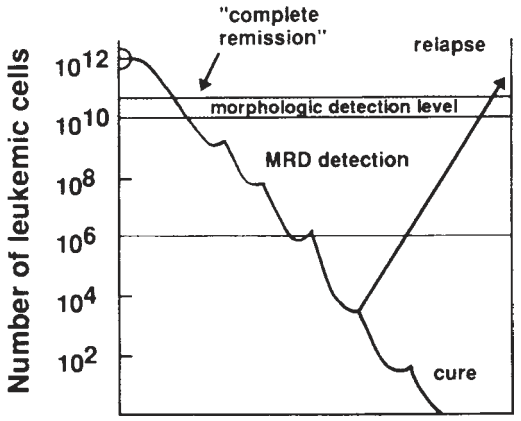

Fig. 1. Schematic representation of leukemic burden, as detected below the sensitivity of the morphologic assessment. Detection of a single leukemic cell among 1×10^4 normal cells is predictive of a subsequent relapse (see text for details). MRD, minimal residual disease.

Table 1
Recurrent Genetic Lesions Useful in Detecting MRD in Acute Leukemias

Cytogenetic subgroup	*Leukemia subtype*[a]	*Molecular alteration (oncoproteins)*
Acute lymphoblastic leukemia		
B-cell precursor ALL		
t(12;21)(p13;q22)	Pro-B	ETV6-AML1
t(1;19)(q23;p13)	Pre-B	E2A-PBX1
t(9;22)(q34;q11)	Pro-B and Pre-B	BCR-ABL (p190 and p210)
t(4;11)(q21;q23)	CD10+ pro-B	MLL-AF4
t(8;14)(q24;q32)	B-ALL	MYC dysregulation
T-ALL		
1p32 deletion		SIL-TAL1
Acute myeloid leukemia		
t(8;21)(q22;q22)	M2	AML1-ETO
inv(16)(p13;q22)	M4Eo	CBF-MYH11
t(15;17)(q22;q11)	M3	PML-RARα

[a]Subclassification of AML follows French-American-British conventions.

Many of these genetic alterations have important prognostic implications that can guide the selection of therapy. In fact, treatment of the acute leukemias has progressed from uniform strategies devised for large groups of patients to more refined protocols tailored to the risk of relapse in discrete subgroups *(10)*. Although routinely recorded features, such as the blast cell immunophenotype and the presenting white blood cell count, provide useful criteria for risk assessment, molecular genetic changes appear to offer the most sensitive markers of potential leukemia cell aggressiveness and hence are the best guides to treatment *(10)*.

PCR analysis of fusion genes is based on the design of oligonucleotide primers at the opposite sides of the breakpoint fusion regions, so that the PCR product contains the tumor-specific fusion sequences (Fig. 2). The precise breakpoint recombination site at the DNA level is specific for each patient and difficult to determine. Thus, the preferred target of PCR analysis is the chimeric mRNA, after its reverse transcription into cDNA (reviewed in ref. *11*).

This approach requires the extraction of total or messenger RNA from bone marrow mononuclear cells, reverse transcription of RNA into cDNA, and molecular assay by PCR, followed by agarose gel electrophoresis. The sensitivity of the method is specific for each target and can be assessed by amplification of serial dilution of diagnostic RNA into RNA from healthy individuals. A single PCR test is sufficiently sensitive (1 leukemic cell in 10^2–10^3 normal cells) to detect fusion transcripts at diagnosis. A higher sensitivity is required for MRD assessment during follow-up analyses and can be achieved by a second round of PCR (nested PCR) using internal primers. In this way 1 leukemic cell in 10^4–10^5 normal cells can be detected for most transcripts. Extra primer sets must be designed to cover fusion gene transcripts with different exon compositions.

Several potential pitfalls must be taken into account to avoid false-positive and false-negative results. First, RNA degradation must be prevented by careful handling. Second, the RT step should be checked by parallel amplification of an appropriate housekeeping gene from the same cDNA preparation tested for fusion genes. Third, a positive sample should be verified by testing an independent sample from the same patient with a different analytic technique.

3.2. Immunoglobulin and T-Cell Receptor Genes

Immunoglobulin (*Ig*) and T-cell receptor γ (*TcR*γ) and δ (*TcR*δ) gene rearrangements are frequently utilized as clonal markers for MRD detection in lymphoproliferative disorders. The latter application is particularly relevant in ALL, since *Ig/TcR* gene rearrangements occur in the vast majority of B-cell precursor ALL patients *(12,13)*. In particular, in ALL, rearrangements of *Ig* and *TcR* genes result in unique recombinations of variable (V), diversity (D), and joining (J) gene segments, and the junctional regions between these gene segments can be regarded as "fingerprint-like" sequences owing to the deletion and random insertion of nucleotides during the rearrangement process *(14)*.

The frequencies and patterns of *TcR* gene rearrangements in ALL were previously addressed by several Southern blot-based studies *(5,15)*. Currently, PCR-based methodologies are more easily and frequently applied to the detection of clonal *TcR* gene rearrangements. Several MRD studies, including two large recent prospective investigations, successfully employed *TcR* gene rearrangements as leukemia-specific PCR targets (reviewed in refs. *5* and *14–18*). Figure 3 shows the outline of the standard procedure. Oligonucleotide primers are designed within consensus sequences of the specific clonal rearrangement, at opposite sides of the junctional region. To discriminate between the leukemia-derived PCR products and PCR products of normal cells with comparable rearrangements, the amplified bands are subjected either to fingerprint *(19)* or homo-heteroduplex

analyses *(20)*. Fingerprint analysis consists of PCR amplification with a fluorescent primer and an electrophoretic run in polyacrylamide gels, in which clonal amplification results in a single peak within a background of polyclonal, constitutional amplification products *(19)*. The homo-heteroduplex analysis takes advantage of the different migration properties in polyacrylamide gel of V-J rearrangements containing a few mismatches (heteroduplex) compared with fully matched V-J junctions (homoduplex) *(20)*.

After the clonal rearrangements are recognized at diagnosis, several methods can be applied to detect the leukemia-derived PCR products specifically, during the follow-up of patients who have undergone therapy. The major variable lies in the sensitivity of the test, which can significantly interfere with interpretation of the assay results.

In the most sensitive assay so far available, clonal PCR products from homo-heteroduplex analysis are directly sequenced. V, D, and J gene segments are then identified, and randomly inserted nucleotides are recognized by comparison with germline sequences in databases. Then the amplification products are hybridized to a patient-specific junctional region probe, which is designed according to the junctional region sequences of the leukemic cells at diagnosis *(14)*. All frozen samples obtained at all time points from a given patient are run at the same time. The specificity of detection is checked for each probe on at least two different polyclonal samples. The sensitivity of each probe is assessed by testing serial dilutions of the patient's blasts in a mixture of polyclonal peripheral blood mononuclear cells. In this way, PCR-based MRD detection via clone-specific junctional regions generally reaches a sensitivity of 1×10^{-4} to 1×10^{-5}.

A less sensitive assay consists of a modified fingerprint analysis, in which the patient- and clone-specific peak corresponding to PCR amplification from residual leukemic cells can be discriminated from normal background, with a sensitivity of 1×10^{-2} *(19)*. The applicability of the allele-specific oligonucleotide (ASO) primer approach depends on its sensitivity and specificity. Factors that influence the specificity of real-time quantitative (RQ)-PCR analysis using an ASO primer are (1) the gene segments used in the junctional regions; (2) the size and sequence of the junctional region; and (3) the background of normal cells with comparable gene rearrangements. Positioning the 3'-end of the ASO primer at the junctional region limits the number of nonspecific amplifications found with most gene sequence combinations under standard RQ-PCR conditions.

4. MRD QUANTITATION BY REAL-TIME RT-PCR IN ACUTE LEUKEMIA PATIENTS

Until now, most PCR-based MRD studies used semiquantitative methods for the detection of either clone-specific translocations *(5,6)* or *Ig* and *TcR* gene rearrangements *(17,18)*. The standard PCR technique has the ability to amplify target DNA up to a plateau, implying that after 35–40 cycles it is not possible to define the initial amount of target DNA precisely. Also, more quantitative methods, such as competitive PCR and limiting dilution, are based on post-PCR end-point analysis *(21,22)*. These techniques require serial dilutions and the analysis of multiple replicates, both of which introduce variability and are difficult and time-consuming to be performed routinely.

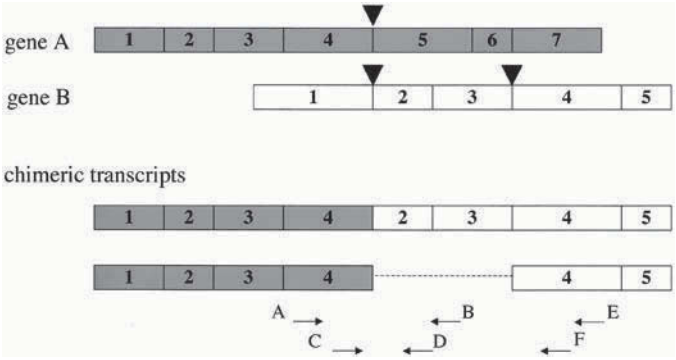

Fig. 2. Schematic diagram of the exonic structure of two potential genes (upper half) involved in a translocation, resulting in a chimeric transcript (lower half). Breakpoint cluster regions are indicated by arrowheads. Alternative chimeric variants are shown. Two possible primer designs for RT-PCR detection of fusion gene transcripts, depending on the exon composition of the fusion gene partners, are depicted. In principle, two external primers (A and B) and two internal primers (C and D) are designed. If more than one breakpoint cluster region occurs, extra E and F primers are designed.

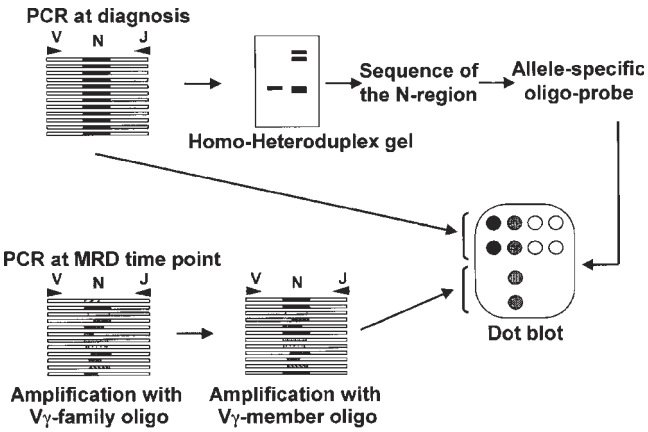

Fig. 3. Schematic outline of the standard semiquantitative procedure for MRD detection (see text for details). PCR, polymerase chain reaction; MRD, minimal residual disease.

The novel RQ-PCR technology circumvents the above problems, because the PCR product accumulation is monitored throughout the complete PCR process *(23)*. The TaqMan technology (ABI 7700 machine; Applied Biosystems, Foster City, CA) is based on the 5'-3' nuclease activity of Taq DNA polymerase and an internal dual-labeled fluorogenic probe with a 5'-reporter dye and a 3'-quencher dye (Fig. 4). During PCR, the 5'-3' nuclease activity of Taq DNA polymerase cleaves the hybridized probe, thereby separating the reporter dye from the quencher dye, resulting in emission of a fluorescent signal that increases during each subsequent PCR cycle. The real-time detection of fluorescence intensity generates quantitative data based on the early cycles of PCR, when the fidelity of amplification is the highest. This quantification can be performed over a large dynamic range of four to five orders of magnitude.

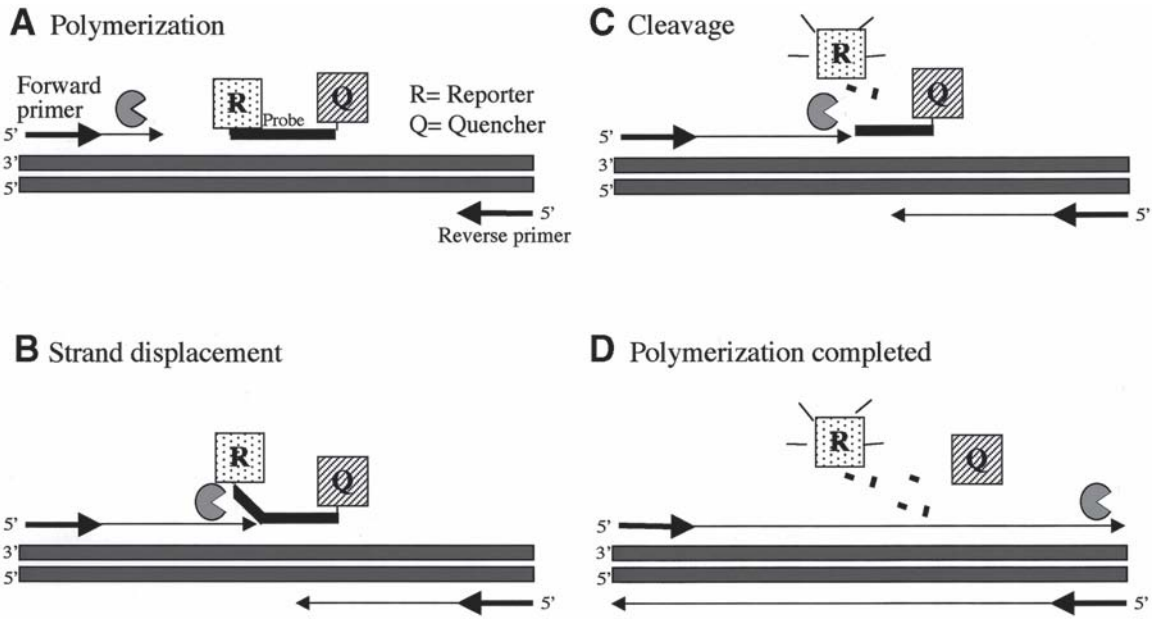

Fig. 4. Principles of the fluorogenic 5' assay ("TaqMan" technology).

This approach has several advantages by comparison with classical end-point quantitative methods. TaqMan technology relies on hybridization probes, which confer to the method a high degree of specificity, without the need to analyze PCR products on a gel with subsequent transfer and hybridization, a time-consuming step with a high risk of interassay contamination. Moreover, compared with the standard end-point methods in which large variations can occur, the method is already known for its efficient reproducibility *(24–26)*. Finally, the 96-well reaction plate provides a convenient tool for simultaneous standard and patient sample testing.

Other methods have been applied to the TaqMan to improve MRD detection. SYBR Green dye gives a bright fluorescence when bound to double-stranded (ds) nucleic acids and is used to monitor nucleic acid amplification *(27)*. However, SYBR Green detects all dsDNA, including primer dimers and other undesired products, and does not allow any verification of product identity. Therefore, the specificity of detection depends only on the specificity of amplification. Because of the demands for specific absolute quantification, SYBR Green should not be used as detection dye for MRD quantification, although it is very useful for establishing PCR-based analyses. When coupled to SYBR Green, melting curve analysis is helpful in avoiding detection of nonspecific amplification through use of clone-specific fluorescent probes for real-time detection *(28)*. Melting curve analysis reveals nonspecific binding of the probe by its lower denaturation temperature and helps to optimize the detection temperature to a level at which no nonspecific binding of the probe is seen.

Other machines are now available for RQ-PCR. Of particular interest is the Light Cycler (LC; Roche, Mannheim, Germany), which has demonstrated potential to quantify MRD *(29–31)*. The most compelling feature of LC technology lies in its combination of rapid thermocycling conditions (owing to the very thin glass capillaries employed for the PCR assay) with on-line real-time fluorescence detection of PCR product amplification. In addition to the TaqMan probes approach and SYBR Green/melting curve analysis, hybridization probes can be used with the LC *(29–31)*. Such probes consist of a pair of neighboring sequences containing different fluorophores at their adjacent ends. The detection of PCR product amplification with hybridization probes is based on fluorescence resonance energy transfer between the fluorophores, a sensitive monitor for their vicinity *(31)*. Moreover, internally labeled general or patient-specific primers can be substituted for one of the hybridization probes. A major advantage of the LC machine is the extreme rapidity of analyses, allowing PCR detection in half an hour without any decreases in sensitivity and reproducibility. By contrast, the (so far) low processing capacity (only 32 samples can be analyzed at once) represents a disadvantage, particularly when the analysis requires contemporary amplification of an adequate number of control samples.

This elegant, flexible, and rapid methodology can be a valid alternative to the TaqMan when only small numbers of samples are being processed, or it can be useful for fast preliminary testing of PCR systems to run in the robust ABI 7700 machine.

4.1. MRD Quantitation of Tumor-Specific Translocations

Numerous publications have demonstrated the feasibility of the RQ-PCR approach to quantify chimeric transcripts resulting from chromosomal translocations *(24–26)*. Although the principles of RQ-PCR are the same whether DNA or RNA is being analyzed, the RT step represents the major difference. In fact, it is necessary to correct variations linked to differences in the RNA amount taken for the reaction or, more importantly, in efficiency (or inhibition) during reverse transcription. For this reason, the number of target gene copies has to be normal-

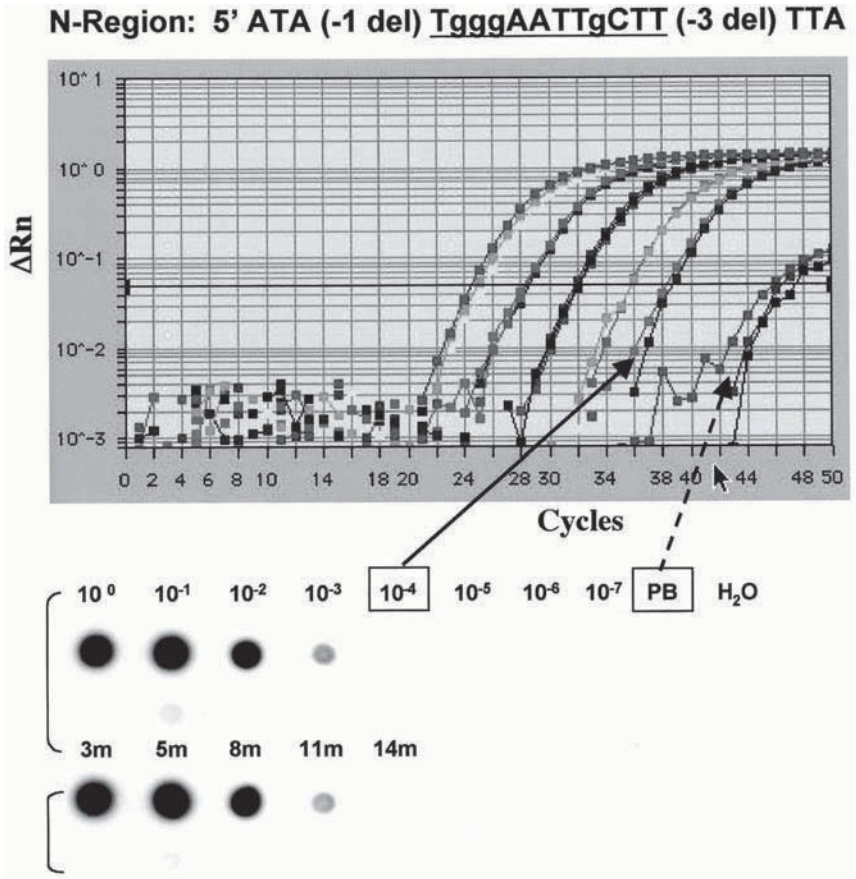

Fig. 5. Sensitivities of the RQ-PCR (upper half) and standard semiquantitative analyses (lower half) are compared by testing serial dilutions of diagnostic DNA (10°) in a pool of DNA from five healthy donors [peripheral blood mononuclear cells (PB)]. The patient-specific, clone-specific sequence of the TcRγ junctional region (N-region) is shown. In this experiment the sensitivity of RQ-PCR (10^{-4}) is 1 log higher than standard analysis (10^{-3}). Amplification from PB is visible at late cycles and does not interfere with the analysis. MRD analysis at various time points was performed only by standard semiquantitative analysis and is shown in the lowest part of the graph (m, month). ΔRn, fluorescence intensity.

ized using a ubiquitously expressed housekeeping gene as a reference (e.g., *ABL*, *b2M*, and *PBGD*) *(24–26)*. Thus, the number of chimeric transcripts will be expressed according to the number of copies of the reference gene transcripts.

As for the classical RT-PCR method, the specificity of RQ-PCR amplification is provided by the localization of PCR primers. Each primer of both partner transcripts is positioned on an exon near the breakpoint. The fluorescent probe is located in a region convenient to cover all of the transcript variants due to different breakpoints and/or alternative splicing. Furthermore, to avoid a contaminating signal from genomic DNA, the primers for the control gene are located in different exons, and the probe is located on the junction between two exons.

4.2. MRD Quantitation of *Ig/TcR* Gene Rearrangements

RQ-PCR can be used to quantify MRD levels with *Ig/TcR* gene rearrangements as PCR targets with ASO probes. Sensitivities of 1×10^{-3} to 1×10^{-5}, comparable to results with the dot-blot method (Fig. 5) *(32,33)*, are achievable with this strategy. Initially, tests were conducted by positioning the fluorescent probe on the junctional region *(31)*. A more useful approach consists of positioning the fluorescent probe and one of the primers at germline *JH TcR* gene segments, in combination with an ASO primer complementary to the junctional region *(32–34)*. This ASO primer approach theoretically results in more sensitive MRD detection compared with use of germline primers, because no competition can occur with the amplification of similar rearrangements in normal cells. Although specific amplification can be easily discriminated from incidental nonspecific amplification, additional stringent conditions may need to be applied to overcome such amplification without affecting the efficiency of the method.

The design of germline fluorescent probes is more cost-effective, since they do not need to vary from patient to patient, but only by type of *Ig/TcR* gene rearrangement. The germline JH fluorescent probes in combination with JH-specific reverse primers might also be useful for quantification of *IgH* gene rearrangements in mature B-cell malignancies, although in most of these cases the presence of somatic hypermutations requires the design of two patient-specific primers *(35)*. In addition, JH fluorescent probes and primers might be useful for MRD detection via chromosomal translocations in which JH gene segments are involved, including t(14;18) and t(11;14) *(36)*.

A disadvantage of this method is that not all junctional regions are suitable for designing a specific ASO primer, particu-

larly in the case of a limited insertion of N-nucleotides. In those cases in which nonspecific amplification still occurs after optimization, it is frequently possible to use another PCR target.

In conclusion, a limited set of germline JH/TcR fluorescent probes and the corresponding germline primers can be used to develop patient-specific RQ-PCR assays, which allow accurate and sensitive MRD analysis in almost all patients with *IgH/TcR* gene rearrangements *(34)*. These results will facilitate standardized RQ-PCR analysis for MRD detection in large clinical studies.

5. NEED FOR STANDARDIZATION TO SUPPORT CLINICAL STUDIES

Although over the last decade numerous methodologies to monitor MRD in acute leukemias have been developed *(2,5,6)* and new technologies are now available *(23,29)*, standardization and quality control are still needed to apply molecular diagnostic procedures in onco-hematology. This is particularly true in efforts to ensure reproducible results within multicenter international studies.

Careful standardization and quality control of MRD techniques were the aims of the European BIOMED-I Concerted Action (Investigation of Minimal Residual Disease in Acute Leukemia: International, Standardization and Clinical Evaluation), with participants from several laboratories in eight European countries *(37)*. Standardization and quality control were performed for the three main types of MRD techniques: flow cytometric immunophenotyping *(38)*, RT-PCR analysis of well-defined chromosome aberrations with fusion gene transcripts *(11)*, and PCR analysis of antigen receptor genes *(39)*.

In particular, nine more frequent, well-defined chromosome aberrations with fusion gene transcripts were selected: t(1;19) with *E2A-PBX1*, t(4;11) with *MLL-AF4*, t(8;21) with *AML1-ETO*, t(9;22) with *BCR-ABL* (p190 and p210), t(12;21) with *TEL-AML1*, t(15;17) with *PML-RARα*, inv(16) with *CBFβ-MYH11*, and microdeletion 1p32 with *SIL-TAL1 (11)*. PCR primers, positioned in such a way as to cover several different transcript versions, were designed according to predefined criteria for single and nested PCR. Additional primers were designed for performing an independent PCR for confirmation or exclusion of positive results (shifted PCR). Various local RT and PCR protocols were compared, and subsequently a common protocol was designed, tested, and adapted, resulting in a standardized RT-PCR protocol. After initial testing, adaptation, and preliminary approval, the primers were tested by all participating laboratories, using cell lines and positive samples as positive controls. Experiments on serial dilutions of positive samples allowed sensitivity testing of each assay. The collaborative effort resulted in standardized primer sets with a minimal target sensitivity of 1×10^{-2} for all single PCR and 1×10^{-4} for all nested PCR analyses. The exchange of experience among laboratories, the central production of reagents, and the regular meeting played an important role in the successful completion of this European Concerted Action, which resulted in a unique, standardized RT-PCR protocol and primer sets that can be used for molecular classification of acute leukemia at diagnosis and for MRD detection during follow-up.

The molecular approach via antigen receptor gene rearrangements also requires standardization and quality control, which has been addressed within the same BIOMED-I framework *(39)*. One of the major problems resides in the fact that the rearrangement is patient-specific and then requires individual optimization, a major difficulty in transferring this methodology to clinical practice. In an effort to cover virtually all ALL patients and to prevent false-negative results, study PCR primers were designed for multiple targets: *TcRδ*, *TcRγ*, and *Igκ* rearrangements. The *SIL/TAL* rearrangement was also included in this study. By following a similar approach to the above-reported standardization of RT-PCR protocols for fusion genes, it was possible to develop primers and protocols for PCR detection and monitoring of antigen receptor rearrangements. In all, 54 primers were designed and approved. These primers can be used with ASO probes in single or nested PCR for target identification at diagnosis, sequence analysis of junctional regions, and MRD detection in follow-up samples. Twenty-five PCR reactions were performed at diagnosis to identify the PCR targets. This standardized approach allowed rapid detection of clonal *Ig/TcR* gene rearrangements in ALL with high sensitivity and high specificity and enabled discrimination between mono- and oligoclonal gene rearrangements. The combination of the four PCR target types allowed PCR monitoring in >90% of B-cell precursor ALL and 95% of T-ALL cases. In the vast majority of childhood and adult ALL cases, two or more PCR targets were available for MRD monitoring. Sensitivity for detecting PCR targets depended at least partially on the size of the junctional region, with a level of 1×10^{-4} reached in most cases.

More recently, a Europe Against Cancer program was established to achieve standardization and quality control for the new RQ-PCR technique for detection and quantification of fusion gene transcripts *(40)*. Twenty-five European laboratories in 10 countries are collaborating to establish consensus standards for RQ-PCR (TaqMan technology) for the main translocations affecting patients with chronic myloid leukemia or acute promyelocytic leukemia (APL), ALL, and AML. The network is organized into nine fusion gene (FG) group [*BCR/ABL* (MBCR and mBCR variants), *MLL/AF4*, *PML/RARα*, *CBFβ/MYH11*, *E2A/PBX1*, *TEL/AML1*, *SIL/TAL*, and *AML1/ETO*, corresponding to 15 targets with their variants], and one group for the ubiquitous genes. Three phases were scheduled: training in experimentation with RNA from cell line dilutions, optimization per fusion gene, and overall sensitivity testing and quality controls. A standardized protocol has been approved for the RT and the PCR steps. For the ubiquitous genes, one highly expressed gene (*β2 microglobulin*) and three modestly expressed genes (*Abelson*, *Cyclophiline*, and *GUS*) have been selected, primarily on the basis of their comparable level of expression in bone marrow vs blood and leukemic vs normal samples. The FG groups selected 12 primer sets and 9 probes to cover the most frequent chimeric transcripts. The threshold of detection of 100 molecules and/or 1×10^{-4} dilution was reached for all the targets by most participants. The first round of quality control included 47 blind samples covering 12 different targets; none showed any contamination during testing.

This work is the first coordinated international effort on standardization and quality control methods for a molecular

diagnosis procedure in onco-hematology across therapeutic protocols. It should allow accurate quantitative measurement of fusion transcripts with an international consensus protocol in diagnosis and follow-up samples from leukemia patients.

6. MOLECULAR DETECTION OF MRD AND CLINICAL STUDIES

The study of MRD has drawn great interest in clinical oncology because of the potential for tailoring treatment and the possibility of gaining insight into the nature of a cure. Several parameters are critical for the interpretation of MRD studies, including the type of disease (ALL or AML), therapeutic context, timing of sampling, target gene, sensitivity of the PCR assay, interlaboratory standardization (particularly relevant in multicenter studies), retrospective or prospective nature of the study, and number of tests conducted for each patient.

6.1. MRD in ALL: A New Tool for Risk Classification?

A prerequisite for applying MRD measurements in clinical studies is that the data should be available for all patients. In ALL, PCR-based MRD detection with *Ig* and *TcR* gene rearrangements can be applied in more than 90–95% of childhood and adult ALL cases. Accordingly, most of the clinical studies of MRD in ALL have used one of the different PCR approaches for the detection of antigen-receptor gene rearrangements *(41)*.

Several retrospective and limited prospective studies indicate that the detection of MRD in childhood ALL has prognostic value, although the results of these studies are not fully concordant. In a recent extensive review of MRD analysis performed in 856 children with ALL and published between 1994 and 1998, Foroni et al. *(5)* showed that approx 50% of childhood ALL cases are MRD-positive at the end of induction treatment, irrespective of the technical approach, and that 45% of those cases will relapse. The absence of residual disease after remission induction is associated with a good prognosis. Only a minority (7.5%) of the patients with negative MRD tests post induction relapsed. The association between a negative test at the end of induction and achievement of CCR is stronger than the association of a positive test with relapse *(5)*. Although results were limited to small series of patients, MRD levels at the end of induction did not seem to be accurate indicators of clinical outcome in adult ALL *(42)*. If multiple bone marrow samples are analyzed during follow-up, a steady decrease of MRD levels to undetectable is observed, with slower decreases in adult compared with childhood ALL patients. In both age groups, the persistence of residual blasts beyond 4–6 mo or the reemergence of residual disease, even at the level of 1×10^{-4}, predicts clinical relapse.

More recently, several prospective studies in childhood ALL have shown that MRD analysis by molecular *(17,18,43,44)* or highly sensitive immunologic methods *(45)*, can predict outcome on the basis of the reduction of the leukemic cell burden during the first months of therapy. A multicenter study performed by the International Berlin–Frankfurt–Münster Study Group (I-BFM-SG) in 240 children with ALL showed that the combined MRD information collected after consolidation therapy identifies three different risk groups according to MRD level *(18)*. Low-risk MRD (degree of MRD, $<1 \times 10^{-4}$) comprises 43% of the patients, whose 3-yr relapse rate was only 2%; by contrast, 15% of the cases with a high degree of MRD ($>1 \times 10^{-2}$) had a relapse rate of 75%. The remaining patients (43%) were in an intermediate-risk group, with a 3-yr relapse rate of 23%. This study suggested that accurate quantification of MRD levels is a critical component of sound clinical management *(46)*. These findings were challenged by the observation of Roberts et al. *(16)*, who reported the frequent persistence of residual disease throughout and beyond the period of treatment, despite CCR, in childhood ALL patients. Higher sensitivity of the MRD detection method used by this group, as well as the presence of an in vitro enrichment step prior to DNA amplification, might account for the discrepant results.

In two large European studies *(17,18)*, MRD positivity was a strong prognostic factor, independent of clinical (age, liver, and spleen size) and biologic (leukocyte or blast count, immunophenotype, DNA index, and chromosomal abnormalities) features commonly used to assess risk status in ALL patients. Even within the medium-risk group, which comprises more than half of newly diagnosed ALL patients and also the largest number of unpredictable relapses, MRD appears to identify patients with different clinical outcomes *(47)*. Significant differences in the results of MRD analysis have been reported to occur in patients with T-ALL compared with those with B-cell precursor ALL *(48,49)*. At most time points the frequency of MRD-positive patients, as well as the MRD levels, was higher in the T-ALL group, reflecting the greater aggressiveness of T-ALL. More recently, the impact of MRD measurements was shown to differ significantly between T-ALL and B-cell precursor ALL patients at early time points (end of induction and just before the start of consolidation treatment). For each 10-fold decrease in MRD level, approx 80% fewer relapses were found in the T-ALL group, compared with approx 60% fewer relapses in the B-cell precursor ALL group *(50)*. Finally, the value of MRD detection at the end of treatment is questionable, because a negative result does not preclude subsequent relapse *(18,45)*, thus reducing the utility of MRD detection as a criterion for elective cessation of treatment.

Now that the cure rates of childhood ALL are approaching 80%, the challenge will be how to incorporate the new MRD information into new studies that pose a therapeutic question. Highly sensitive PCR techniques (detection limit $1 \times <10^{-4}$) allow the identification of a significant proportion of ALL cases with excellent clinical outcomes in the presence of negative MRD findings at early time points in treatment. By contrast, patients with $\geq 10^{-2}$ leukemic cells during any phase of remission induction should be regarded as having a very high risk of relapse, thus becoming eligible for early transplantation or experimental treatment. How to use the "intermediate" range of positive MRD findings ($>1 \times 10^{-4}$ but $<1 \times 10^{-2}$) is still unclear. Such patients might benefit from further intensification, but that possibility needs to be substantiated by randomized clinical studies. Thus, the German BFM and the Italian Association for Pediatric Hematology and Oncology (AIEOP) study groups have adopted an MRD-based risk group classification for treatment stratification in their ongoing clinical studies. It is hoped that a more sensitive and specific evaluation of remission and early response to treatment could speed further improvement in cure rates for children with ALL.

6.2. Heterogeneity Among Genetically Homogeneous ALL Subgroups: Is It Clinically Relevant?

RT-PCR of fusion transcripts generated by t(9;22), t(4;11), t(1;19), and t(12;21) has been used to assess MRD. The data reported so far have been controversial, leaving unanswered the question of whether MRD assessment based on tumor-specific translocations would help to identify patients with different outcomes within the same genetically defined subgroup.

PCR positivity almost invariably persists in chemotherapy-treated patients with ALL bearing t(9;22) and correlates with the high relapse rate of this disease subtype in both adults and children. However, more recent MRD studies following intensive combination chemotherapy (without transplantation) provide intriguing preliminary evidence that achievement of a *BCR/ABL*-negative state, as detected with PCR, may predict durable remissions in Philadelphia chromosome-positive (Ph+) ALL in adults *(51)*. Along the same lines, in a retrospective study *(52)* and more recently in a larger series *(53)* of children, Ph+ ALL appeared to be quite heterogeneous with regard to treatment sensitivity. To investigate the clinical relevance of molecular monitoring of Ph+ ALL, we have prospectively evaluated all patients with t(9;22) enrolled in the ALL-AIEOP 95 study, using a highly sensitive RT-PCR method *(11)*. The results reveal considerable heterogeneity with respect to MRD levels, even within the Ph+ ALL subgroup with good early responses to steroids *(54)*. Thus, MRD evaluation could help to identify patients persistently negative for the *BCR/ABL* fusion gene among those with good early responses to treatment who can be cured with intensive chemotherapy alone.

Using primer sets from the *MLL* and *AF4* oncogenes, a number of investigators have studied a limited number of cases *(55–57)*. These results indicate that early conversion to or persistent PCR negativity (particularly after 3 mo) is associated with prolonged CCR. Similar results were recently confirmed in a prospective series of t(4;11)-positive ALL patients *(58)*.

As reported by Foroni et al. *(5)* in an extensive review, MRD analysis of ALL patients with the t(1;19) translocation has been performed in 73 children and three adults *(5)*. Although the vast majority of patients achieved a molecular remission, MRD persisted as long as 8–12 mo (in 25 patients) or 24–27 mo or longer (in 4 patients) without the occurrence of clinical relapse *(59–61)*. This indicates that persistently negative tests are a good indicator of CCR but that positive tests are not necessarily an accurate predictor of relapse, thus reducing the utility of this test for clinical decision making. It is likely that continuous monitoring with accurate quantification may represent the most reliable approach.

Residual disease has been investigated in a limited number of patients with ALL carrying t(12.21) *(62–65)*. Several cases tested positive between 2 and 4 mo post induction, but relapse was observed in cases with persistent MRD positivity $>1 \times 10^{-3}$. However, relapse has been reported to occur even in patients with previous negative tests. Larger prospective studies are needed to assess the prognostic value fully of the t(12;21) translocation as well as its value as a marker for monitoring MRD in childhood ALL.

6.3. MRD in Acute Promyelocytic Leukemia: Tool for Definition of Molecular Relapse?

The APL phenotype is associated with chromosomal translocations disrupting the *RARα* locus and resulting in fusions with other genes encoding nuclear proteins (*PML/RARα*, *PLZF/RARα*, *NuMA/RARα*, or *NPM/RARα*; reviewed in ref. *66*). Despite their similar clinical correlates, all-*trans*-retinoic acid (ATRA) induces differentiation and clinical remission in only *PML/RARα*-positive APL, whereas *PLZF/RARα*-positive APL is ATRA resistant. The exquisite sensitivity to ATRA of *PML/RARα*-positive APL has made demonstration of the tumor-specific fusion gene a mandatory step in the proper diagnosis of this AML subtype *(67)*. Among the different diagnostic methods new available [conventional karyotyping, FISH, and PML immunostaining with specific antibodies *(68)*], only RT-PCR detection of the *PML/RARα* fusion gene appears suitable for MRD detection *(69)*.

There is general agreement that a positive *PML/RARα* test after consolidation therapy is a strong predictor of subsequent hematologic relapse, whereas repeatedly negative results are associated with long-term survival in most patients. However, these correlations are not absolute, being reported in patients who remain PCR-positive in long-term remission or, more often, who ultimately relapse after negative tests (reviewed in refs. *67* and *70*)

In the three large prospective studies of APL patients treated with ATRA and chemotherapy in whom molecular diagnosis and monitoring have been assessed *(71–73)*, the persistence of *PML/RARα* fusion transcripts following completion of therapy was predictive of relapse. Nevertheless, most patients who ultimately relapse lack PCR evidence of MRD in the marrow at the end of therapy. These findings support the notion that achievement of PCR negativity in APL cannot be equated with cure and underscore the relative insensitivity of the RT-PCR *PML/RARα* assay. In dilution experiments, we and others were able to detect (*PML/RARα* transcripts) in concentrations as low as 50 pg of total RNA, but only 1 in 1×10^{-4} *PML/RARα*-positive cells. This means that the assay is approx 1 log less sensitive than the RT-PCR assay applied to different chimeric genes generated by other chromosomal abnormalities, such as the *BCR/ABL* in t(9;22)-positive leukemias .

Why, then, do we need to monitor APL patients with molecular assays? The advent of ATRA therapy has led to a dramatic improvement in survival among patients with APL, such that the relapse risk has decreased to 10–20% *(70–72)*. The current challenge is how to identify the relatively small subgroup of patients at particularly high risk of relapse whose prognosis cannot be gleaned from pretreatment characteristics but who may benefit from more intensive treatment in first remission.

The Gruppo Italiano Malattie Ematologiche Maligne Adulto (GIMEMA) group reported that recurrence of PCR positivity, detected by 3-mo surveillance of marrows performed after completion of therapy, was highly predictive of relapse *(74)*. Using such a strategy, these investigators accurately predicted relapse in approx 70% of cases. Clinical relapse occurred at a median of 3 mo from the detection of molecular relapse. These findings are of great relevance in

view of the ATRA trial reported by the Medical Research Council (MRC), emphasizing the poor prognosis of patients relapsing after first-line therapy with ATRA and other agents *(72)*.

Another perspective on the use of MRD to identify APL patients at higher risk for relapse emphasized the kinetics of achievement of molecular remission. In view of the relatively low sensitivity of the *PML/RARα* assay, monitoring of the reciprocal *RARα/PML* fusion gene, which is expressed in most but not all patients, was proposed as being at least 1 log more sensitive (1 cell in 1×10^5) *(75)*. The *RARα/PML* assay led to the detection of residual disease in an additional 20% of patients in morphologic remission. Nevertheless, even APL patients with an informative *RARα/PML* assay who ultimately relapsed had no molecular evidence of MRD at the end of therapy.

Although the molecular diagnosis and monitoring of APL represents one of the most relevant examples of the impact of molecular genetics in clinical hematology, further investigations are still needed. Would we benefit from a more sensitive RT-PCR assay for *PML/RARα* in the identification of patients at higher risk for relapse? Is quantitative PCR useful for earlier monitoring? Is it of benefit for the patient treated at the stage of molecular relapse?

6.4. AML with t(8;21) and inv(16): "Stable MRD" or Full Eradication for Cure?

Compared with assays based on the *PML/RARα* fusion mRNA, methods for the detection of t(8;21)/ *AML1/ETO* and inv(16)/*CBFβ/MYH11* seem to be more sensitive *(6)*. Studies on the use of RT-PCR for molecular detection of t(8;21) have yielded controversial results. Using sensitive RT-PCR methods, several groups have reported the persistence of *AML1/ETO* fusion transcripts in patients in complete remission for as long as 94 mo *(76–78)*. Moreover, persistence of residual disease has also been reported in patients undergoing autologous or allogeneic bone marrow transplantation *(78,79)*. In contrast to these findings, other authors reported the absence of *AMLI/ETO* transcripts in a significant number of long-term remitters *(80,81)*. Reported experience with molecular monitoring of patients carrying inv(16) is quite limited. Nevertheless, some AML/inv(16) patients in CCR may convert to an RT-PCR-negative status *(21)*.

These marked discrepancies may be explained on the basis of different methods of MRD analysis with variable sensitivities and the biologic diversity of the different leukemia subtypes. Differences in the clonogenicity and aggressiveness of leukemic blast cells from individual cases could also play a role, as could the requirement for additional mutations to induce overt leukemia. Alternatively, as in the case of t(8;21), cell proliferation may be repressed by an unknown mechanism(s). Quantitative RT-PCR methods are likely to help in defining the critical threshold of transcripts above or below which one can predict impending relapse or continuing remission. Confirmation that cure can be achieved even in the presence of long-term "stable MRD" might offer unique opportunities to investigate mechanism(s) responsible for disease control in the absence of chemotherapy.

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Treatment of Acute Leukemias
New Directions for Clinical Research

Edited by

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Although much progress has been made in the treatment of acute leukemia, there is still vigorous debate over the optimal methods for securing additional gains in event-free survival and for reducing the toxic side effects of "curative" therapy. In *Treatment of Acute Leukemias: New Directions for Clinical Research,* international experts not only review the state-of-the-art in managing children and adults with acute leukemia, but also debate the pros and cons of current controversial and problematic issues. The book summarizes the best diagnostic and treatment practices for newly diagnosed and relapsed acute lymphoblastic and myeloid leukemia in children, adolescents, and adults. Among the therapies considered are methotrexate, asparaginase, antipurines, epipodophyllotoxins, hematopoietic stem cell transplantation, hematopoietic growth factors, and immunotherapy. Problems of central-nervous-system therapy, minimal residual disease, drug resistance, and directions of future research are also considered. Discussion of each major topic by two distinguished groups of investigators ensures broad and balanced coverage.

Comprehensive and up-to-date, *Treatment of Acute Leukemias: New Directions for Clinical Research* offers oncologists practical guidelines for precise diagnosis and optimal treatment of childhood and adult acute leukemia, as well as insights into those aspects of patient management most in need of further study and refinement.

- **Review of the best diagnosis and treatment practices for childhood and adult leukemias**
- **Coverage of current controversial and problematic issues in patient management**
- **Discussion of each major topic by two experts sometimes with different views**
- **Insight into those aspects of patient management most in need of further study**

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