
PREFACE

Leukemia and Lymphoma: Detection of Minimal Residual Disease is being published at a time when the detection of microscopically unobservable disease in the leukemias and lymphomas is leaving its adolescent stage and entering the early stages of maturity. Detection of disease at levels almost four orders of magnitude below detection by light microscopy has now been accomplished and many of the methods have been shown to provide reproducible results. The groundwork has thus been laid for future construction of a methodology superior to the present methods of outcome prediction. Results of this nature will undoubtedly be necessary to justify the expense of future large-scale clinical trials using the various minimal residual disease (MRD) techniques that have been developed during the past decade. This places the burden of proof on those clinical investigators, statisticians, and basic scientists who are convinced that such measurements have an important role in producing future advances in treatment outcome.

As editors we have chosen contributors who have been successful in applying their chosen technique to the particular diseases that are their interests. We have especially tried to select those authors who were responsible for developments in the technique they used to make residual disease measurements. For this reason, we have encouraged them to give their insights into the methodology used in their research. As a result the reader will find several different descriptions of similar laboratory and analysis techniques, each of which we hope will prove helpful. We have taken advantage of the expertise of Professor Ludwig and Dr. Ratei to include a separate chapter on flow cytometry techniques. This addition should facilitate understanding the chapters on the use of flow cytometry to detect residual disease in the lymphoid and myeloid leukemias. In addition, one of us (DAJ) has written a rigorous mathematical description of an approach to the analysis of the predictive capabilities of an MRD detection system that accompanies a clinical trial. There is also a section of Editor's Notes at the end of this volume that contains comments on portions of each chapter. We hope that these comments will be found helpful to readers.

The chapter by Dr. Dario Campana describes the use of a patient-specific immunophenotype to identify residual leukemia in patients who are in remission. He carefully describes the different categories of leukemia-associated immunophenotypes and shows how they are used to follow patients in remission. His research has shown that he can detect one leukemia cell among ten thousand normal marrow cells.

Dr. Geoffrey Neale, a veteran contributor to the study of Childhood Acute Lymphocytic Leukemia using polymerase chain reaction (PCR)-based methods, describes the original methods for the detection of residual disease using

PCR. He then progresses to a discussion of the various methods of quantitation of the level of disease. He completes his chapter with a description of real-time quantitative PCR (RQ-PCR). Throughout the chapter he gives the outcome of clinical studies of MRD in patients during remission.

Following Dr. Johnston's chapter on the analysis of MRD studies, we both have written a brief discussion on the evaluation of these techniques when used in clinical trials. In particular we address the question of assessing the predictive capability of a study that follows patients during and after therapy. We use simple arithmetic methods of performing these evaluations.

The predictive properties of semiquantitative PCR measurements of MRD prior to and after allogeneic stem cell transplantation of children with ALL are described by Moppet et al., who outline their experience with the technique in this setting. This chapter provides the reader with many possible routes for future MRD studies of allogeneic transplantation.

Drs. Foroni, Mortuza, and Hoffbrand give a detailed summary of the high sensitivity monitoring of adult patients with ALL. They review the approaches used to detect residual leukemia during remission. They define high and low risk groups according to the measured response to therapy and also contrast MRD in adult and childhood ALL.

Professor San Miguel and his colleagues present an extensive discussion of the immunophenotypes observed in patients with Acute Myeloid Leukemia (AML) and the frequent occurrence of asynchronous antigen expression in this disease. This chapter gives an excellent presentation of the methods of confirming the detection sensitivity of the flow cytometry-based assay. The results of clinical studies using this method to identify risk groups are presented.

The chapter presented by Drs. Marcucci and Caligiuri discusses the non-random chromosomal abnormalities in AML that lead to chimeric fusion genes thought to be leukemia-specific. Reverse transcription PCR (RT-PCR) is capable of detecting these transcripts with high sensitivity and thereby allows monitoring of the leukemia during remission. The authors review the results of clinical studies that detect these fusion transcripts and the use of their data in stratifying patients during remission according to the risk of relapse. They also discuss the importance of high sensitivity, as well as the associated hidden risks.

Dr. Lo Coco and Ms. Diverio discuss detection of MRD in Acute Promyelocytic Leukemia (APL), a subtype of AML that is associated with a specific chromosomal translocation, the t(15;17). The detection of the reverse transcription product of this fusion gene using RT-PCR has become an important aspect of both the diagnosis and monitoring during remission of this leukemia. The authors present a comprehensive review of the laboratory and clinical aspects of this endeavor.

The detection of MRD in Chronic Myelogenous Leukemia (CML) is described in the chapter of Drs. Cross and Hochhaus. Their discussion begins with the molecular genetics of the CML-associated t(9;22) chromosomal translocation and the BCR-ABL fusion gene. This is followed immediately by a section on the methods used to detect CML cells and leads to the application of RT-PCR to detect the BCR-ABL fusion transcript. The presentation of qualitative RT-PCR to detect the presence of the leukemia-associated transcript is closely followed by a thorough discussion of the methods for quantitation of the number of BCR-ABL transcripts. The chapter concludes with a critical discussion of the results of BCR-ABL detection in patients with CML. The problems associated with defining molecular relapse is presented in addition to a brief discussion about the presence of the BCR-ABL transcript in normal individuals.

Drs. Krackhardt and Gribben describe the detection of the t(14;8), t(11;14), t(8;14), t(2;5), t(11;18), and the antigen receptor gene rearrangements. They then present quantitation strategies based upon competitive PCR. They then apply the PCR technique to detection of these translocations in bone marrow and peripheral blood of patients with Non-Hodgkin's Lymphoma who have been treated with autologous bone marrow stem cell transplantation. The issue of whether or not "molecular complete remission" is the goal of therapy is presented in an unambiguous manner.

Tsimberidou et al. summarize the literature regarding the use of real-time and conventional PCR in patients with Non-Hodgkin's Lymphoma and t(14;18). They then describe real-time PCR, as they apply it in their laboratory, to specimens from patients with follicular lymphoma. They studied peripheral blood and bone marrow in these patients, as well as peripheral blood from normal donors. Their technique employs the simultaneous amplification of an internal beta actin sequence for quantitation and comparison.

Drs. Lee and Cabanillas describe the application of PCR to monitoring follicular lymphoma in patients with all stages of disease during remission. They use PCR results to define molecular nonresponders and develop a multivariate analysis of these patients. There was a high complete remission rate for patients who were molecular responders and a low rate for the nonresponders. They extend this work to patients treated with bone marrow transplantation.

Throughout this book there are several issues that reappear frequently. They represent uncertainties about MRD that must be resolved before these assays achieve status as a reliable tool for clinical decision-making. Since these issues are essentially of equal importance, the following is not in any particular order of impact: (1) Some investigators have observed persistent low levels of detectable disease in patients who remain in clinical remission.

This observation is closely coupled to the question of the optimal (most cost effective?) detection sensitivity. It also raises the question of interference by normal background. (2) The capability of detecting disease at submicroscopic levels has given rise to the real possibility of new definitions of the clinical terms remission and relapse.

These new definitions might allow improvements in treatment outcomes if they were properly established. For example, a reliable definition of molecular remission applied in cases where it was found persistently could lead to decreased treatment morbidity. There are two possible benefits of a reliable definition of molecular relapse. First, the signal for molecular relapse would, optimally, appear when the disease level is quite low and, at this level, the disease may be sensitive to many innovative therapeutic interventions. Second, prior to clinical relapse the patient is probably better able to tolerate intensive therapy than after clinical relapse. The actual implementation of these new criteria would provide an entrée to a completely new area of clinical investigation that could be very beneficial to patient care. (3) Many authors have noted that standardization is necessary. This standardization must include not only laboratory methods, but also the statistical methods used to analyze the data. This is an absolute requirement for the comparison of data from different institutions. (4) Finally, the emergence of the RQ-PCR technique as the method of choice is quite apparent in these chapters and in the recent literature. It seems probable that this development will facilitate the standardization of detection and quantitation techniques. We make these observations here so that the reader may keep them in mind as he/she reads the following chapters. If this book is to have any impact, it will hopefully inspire its readers to find solutions to the problems that now face the field.

The editors would like to thank the authors for the variety of their excellent chapters. More important, we would like to thank them and the many others working with MRD for the lively and impassioned discussions of MRD and their willingness to share their technical methods and ideas for the direction, use, and application of MRD. This has advanced the techniques and applications far beyond what we could have done working individually.

The editors would also like to thank Walter Pagel for his technical assistance and encouragement on this project, and Connie Siefert and Candy Schuenenman for their excellent editorial assistance. And last but most important, we would like to thank our wives, Maureen and Janice, for their support and patience throughout this project.

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Flow-Cytometry–Based Studies of Minimal Residual Disease in Children with Acute Lymphoblastic Leukemia

Dario Campana

INTRODUCTION

A central problem in childhood acute lymphoblastic leukemia (ALL) is the identification of patients who require more aggressive therapy to avert relapse. Although clinical (e.g., white blood cell count, age) and biologic (e.g., immunophenotype, ploidy, structural chromosomal abnormalities, and gene rearrangements) parameters can be used for treatment stratification, none of these prognostic factors is ideal. A proportion of patients with “good risk” features relapse, whereas others may receive more intensive treatment than is necessary. Studies of minimal residual disease (MRD) aim at improving estimates of the total burden of leukemic cells during clinical remission. This information provides an indicator of the aggressiveness and drug sensitivity of the disease and helps in the selection of appropriate therapeutic strategies.

Reliable MRD assays would allow not only the early identification of patients at a higher risk of relapse and detection of impending clinical relapse, but would also provide a powerful tool for assessing bone marrow or peripheral blood that has been harvested for autologous hematopoietic stem cell transplantation and for determining the efficacy of “purging” procedures (1). In addition, MRD measurements could be used as end points to rapidly compare the effectiveness of different chemotherapeutic regimens.

The main purpose of MRD assays is to be clinically useful. Therefore, methods must be robust, reliable, rapid, and suitable for a clinical laboratory. Numerous methods of monitoring MRD in acute leukemia have been developed and are

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discussed extensively in this book. The following sections review the methodologic and clinical advances in the detection and measurement of MRD in childhood ALL based on immunophenotype.

IMMUNOPHENOTYPIC IDENTIFICATION OF LEUKEMIA CELLS

Rationale

In general, the immunophenotype of leukemic cells reflect that of their normal counterparts. The normal equivalents of T-lineage ALL cells are immature T-cells, which expand in the thymus and are confined to this organ (2). Therefore, in patients with this subtype of leukemia, detection of MRD in the bone marrow or in the peripheral blood is relatively straightforward: It consists in the identification of immunophenotypically immature T-cells (3). For example, the combination CD3/TdT is expressed by most T-lineage ALL cells and by developing T-cells in the thymus, but it is never observed among normal peripheral blood or bone marrow cells (4). Other similar thymus-restricted immunophenotypic combinations can also be used (5). In the case of B-lineage ALL cells, the normal equivalent cells are the B-cell progenitors, which normally reside in the bone marrow (6). These cells are particularly abundant in samples from young children or in bone marrow regenerating after chemotherapy and bone marrow transplantation (7) and can also be found, albeit in low proportions, in the peripheral blood (8). Therefore, in patients with this subtype of leukemia, detection of MRD by immunophenotypic criteria depends on the identification of molecules differentially expressed in normal and leukemic cells.

Differentially expressed molecules that constitute leukemia-associated immunophenotypes in B-lineage ALL can be classified in three broad categories. The first category includes the product of gene fusions that accompany chromosomal translocations such as *BCR-ABL*, *E2A-PBX1*, *MLL-AF4*, and *TEL-AML1*. The encoded chimeric proteins are genuine tumor-associated markers, and antibodies specific for these tumor markers should be useful for studies of MRD. Unfortunately, it has been difficult to produce monoclonal antibodies that would allow reliable detection of these proteins by immunofluorescence, although one such reagent, which reacts with the *E2A-PBX1* chimeric protein, has been described (9).

A second category is represented by molecules whose expression becomes dysregulated by the leukemic process. For example, the human homolog of the rat chondroitin sulfate proteoglycan (NG2), recognized by the antibody 7.1, is expressed by leukemic lymphoblasts (generally those with 11q23 abnormalities) but not by normal hematopoietic cells (10,11). Another molecule, CD66c, is expressed in approximately one-third of B-lineage ALL cases, but it is not expressed in normal B-cell progenitors (12–15). Because this antibody also reacts

with myeloid cells, it must be used in combination with reagents that identify lymphoid progenitors.

A third category of immunophenotypic features that can be used to distinguish B-lineage ALL cells from normal B-cell progenitors is represented by molecules that are expressed during normal B-cell development but are relatively underexpressed or overexpressed in leukemic cells (16–18). For example, normal CD19⁺CD34⁺ B-cell progenitors lack CD21, a marker expressed later during differentiation, but cells in a proportion of B-lineage ALL cases are CD19⁺CD34⁺CD21⁺. In addition, a number of quantitative differences in antigen expression can be used to distinguish leukemic blast cells from subsets of normal cells with similar phenotypes. Thus, the expression of CD19, CD10, and CD34 in some cases of B-lineage ALL can be more than 10-fold greater than that of normal B-cell progenitors (16–19). Underexpression of CD45 and CD38 is also an abnormal feature in some B-lineage ALL cases (16–20). Other markers, such as CD45RA, CD11a, and CD44 may also be overexpressed or underexpressed (21). Overexpression of WT1 in ALL (22,23) could also, in principle, be exploited for MRD studies.

Flow Cytometry

In theory, the availability of antibodies to true tumor antigens (e.g., proteins product of gene fusions) would allow identification of residual leukemic cells. Even in this case, however, it seems likely that focusing the analysis on selected cell subsets identified by other markers would increase the reliability and sensitivity of the assay. Current immunologic strategies for detecting residual disease rely on combinations of multiple markers; hence, they cannot be performed by immunohistochemistry. They require immunofluorescence techniques that allow the simultaneous application of antibodies conjugated to different fluorochromes. Early MRD studies were performed by fluorescence microscopy (3–24). However, microscopic screening of a large number of cells is tedious and time-consuming. The automation of this process by computerized image analysis is desirable, but we have not yet encountered a sufficiently sensitive and accurate instrument that we could recommend for reliable MRD detection. Virtually all laboratories currently prefer flow cytometry, which allows multiparameter analysis, antigen quantitation, and rapid screening of large numbers of cells. Additional capabilities of flow cytometry, such as cell sorting followed by fluorescence *in situ* hybridization (FISH) (25) or simultaneous analysis of phenotype and DNA content (26), may also aid MRD studies, but have not been used extensively for this purpose.

Currently, most laboratories use three-color analysis for MRD studies, including antibodies conjugated to FITC, PE, and PerCP. Dual-laser flow cytometers that allow the detection of other fluorochromes such as antigen-presenting cells (APC) and permit four-color analysis appear to be ideally suited for these studies.

In addition, this approach potentially allows a reduction in the number of individual test tubes per sample, thus employing reagents and cells more efficiently.

Sensitivity and Precision

The sensitivity of detecting rare cells by flow cytometry is determined by the degree of difference in the features of the target population as compared to the remaining cells and by the number of cells that can be counted. As discussed in this chapter, ALL cells do express markers in combinations that are not found in normal hematopoietic cells. Thus, the main limitation in sensitivity for all MRD detection methods is the number of cells that can be analyzed. A marrow sample taken from a child with acute leukemia in clinical remission typically yields 5×10^7 or fewer mononuclear cells, and technical constraints may limit the number of cells available for study to less than 1×10^6 . Flow cytometry allows the detection of 1 target cell in 10^8 or more cells, providing that a large number of cells is studied (e.g., 10^8 or more) and the fluidics system is exhaustively cleansed (27–30). Because such large samples are rarely available during routine MRD studies in patients with leukemia, a more reasonable sensitivity for practical applications is approximately 1 target cell in 10^4 – 10^5 cells.

To determine the precision of flow-cytometric detection of rare cells, we prepared mixtures of leukemic and normal cells and compared the results of multiple measurements of residual leukemia in identical cell preparations. The results demonstrated that this assay is highly precise: in 23 tests of mixtures containing 1 leukemic cell in 10^4 normal cells, results were remarkably similar (coefficient of variation = 15%); in 22 tests of mixtures containing 1 leukemic cell in 10^3 cells, the coefficient of variation was 10% (31).

Methodological Approach

The methodological approach that we use in our laboratory has previously been described in detail (16). Briefly, we first perform a detailed analysis of the immunophenotype of the leukemic blasts at diagnosis or at relapse. The results from the patients' cells are then compared to previously obtained results of an identical immunophenotypic analysis of normal bone marrow and peripheral blood samples. The most distinctive marker combinations in each case are thus selected. If the immunophenotype of the leukemic cells were not known, one would have to apply the full range of potentially useful markers for MRD studies in remission samples. This not only would be expensive and time-consuming, but it may still fail to identify residual disease.

At the time of MRD analysis, cells are labeled with the selected antibody combinations, and the light-scattering and immunophenotypic features of 10,000 cells are recorded (16). We then selectively store and examine the information for cells that fulfill the predetermined morphologic and immunophenotypic criteria, from a total of over 2×10^5 bone marrow mononuclear cells.

At least 10–20 dots expressing the leukemia-associated features must be captured to interpret a cluster of abnormal flow cytometric events.

Selection of Markers

To date, leukemia-specific phenotypes have been searched by systematically comparing the immunophenotypes of leukemic cells with those of normal bone marrow cells (16). This approach has identified phenotypic features that are uniquely associated with leukemic cells and are never expressed during normal hematopoietic cell development, even during chemotherapy or after bone marrow transplantation. Unfortunately, this process is slow and largely based on trial and error.

We have recently used cDNA arrays to identify immunophenotypic differences between ALL cells and normal lymphoid progenitors (32). By cDNA array analysis, 334 of 4132 genes studied were expressed 1.5-fold to 5.8-fold higher in leukemic cells relative to both normal samples; 238 of these genes were also overexpressed in the leukemic cell line RS4;11. We selected 9 genes among the 274 overexpressed in at least two leukemic samples and measured expression of the encoded proteins by flow cytometry. Seven proteins (CD58, creatine kinase B, ninjurin1, Ref1, calpastatin, HDJ-2, and annexin VI) were expressed in B-lineage ALL cells at higher levels than in normal CD19⁺CD10⁺ B-cell progenitors ($p < 0.05$ in all comparisons). Because of its abundant and prevalent overexpression, CD58 was chosen for further analysis. An anti-CD58 antibody identified residual leukemic cells (0.01–1.13%, median 0.03%) in 9 of 104 bone marrow samples from children with ALL in clinical remission. MRD estimates by CD58 staining correlated well with those of polymerase chain reaction (PCR) amplification of immunoglobulin genes.

The identification of immunophenotypic differences between normal bone marrow cells and leukemic cells in diagnostic samples is just a starting point. It is then important to test the expression of the selected markers on normal bone marrow cells under different conditions. In particular, it is crucial to establish whether levels of expression remain consistent in leukemic cells of patients undergoing chemotherapy and in normal cells actively proliferating after chemotherapy or bone marrow transplantation. Investigators planning to test samples after several hours from collection (e.g., those shipped from other centers) should ensure that the leukemia-associated immunophenotypes are stable. In addition, experiments with mixtures of leukemic and normal cells are required to test the sensitivity afforded by the new immunophenotypic combination.

ANTIBODY PANELS

Table 1 summarizes the phenotypic combinations currently used in our laboratory. We use only immunophenotypes that allow us to detect 1 leukemic cell

Table 1
Immunophenotypic Markers Currently Used to Study MRD in Children with ALL

<i>ALL lineage</i>	<i>Type of phenotypic abnormality</i>	<i>Markers</i>	<i>Frequency (%)^a</i>
B	Overexpression or under expression of markers also expressed in normal B-cell progenitors	CD19/CD34/CD10/TdT	30–50
		CD19/CD34/CD10/CD22	20–30
		CD19/CD34/CD10/CD38	30–50
		CD19/CD34/CD10/CD45	30–50
		CD19/CD34/CD10/CD58	40–60
		CD19/CD34/CD10/ CD13	10–20
	Expression of markers not expressed in normal B-cell progenitors (aberrant marker)	CD19/CD34/CD10/ CD15	5–10
		CD19/CD34/CD10/ CD33	5–10
		CD19/CD34/CD10/ CD65	5–10
		CD19/CD34/CD10/ CD56	5–10
		CD19/CD34/CD10/ CD66c	10–20
	Expression of markers expressed at different stages of normal B-cell maturation	CD19/CD34/CD10/ 7.1	3–5
		CD19/CD34/CD10/CD21	5–10
		CD19/CD34/TdT/cytopl.μ	10–20
T	Phenotypes normally confined to the thymus	TdT/CD3	90–95
		CD34/CD3	30–50

^aProportion of childhood ALL cases in which 1 leukemic cell in 10⁴ normal bone marrow cells can be detected with the listed immunophenotypic combination. Most cases express more than one combination useful for MRD studies (16).

in 10⁴ or more normal cells. We use the combination of nuclear TdT with T-cell markers, such as cytoplasmic or surface CD3 or CD5, in virtually all cases of T-ALL. In cases with weak or negative TdT expression, we used CD34 instead, if this marker is found to be expressed at diagnosis. CD19 and HLA-Dr, which are not usually expressed on T-ALL blasts but are strongly positive on most normal bone marrow TdT⁺ cells, can be used to further distinguish normal from leukemic cells. By this approach, MRD can be studied in virtually all cases of T-ALL.

Detection of MRD in B-lineage ALL requires a larger panel of antibodies. We usually identify immature B-cells by the simultaneous expression of CD19, CD10, and CD34 or TdT. Quantitative differences in antigenic expression between leukemic and normal cells in the expression of these markers can be used in approx 30–50% of cases. Other useful markers whose expression may differ quantitatively in leukemic and normal immature B-cells are CD38, CD45, CD22, and the recently identified CD58. Qualitative differences between normal and leukemic cells can be detected by using antibodies to myeloid- and NK-associated molecules or to molecules expressed by mature normal B-cells.

CD66c and anti-NG2 (7.1), as mentioned above, are two additional informative markers. With all of these marker combinations, approx 90% of B-lineage ALL cases can be studied at the 1 in 10^4 level of sensitivity.

Flow Cytometry Compared to PCR

Each method of MRD detection has specific advantages and potential pitfalls (33). For example, immunologic techniques yield a more accurate quantitation of MRD and can discriminate viable from dying cells, whereas PCR may have superior sensitivity. In any case, neither immunologic nor molecular techniques can, at present, be applied to all patients, which is a prerequisite for the introduction of MRD monitoring in clinical protocols. To determine how well measurements obtained by flow cytometry and PCR amplification of IgH genes were correlated, we tested serial dilutions of normal and leukemic cells by both methods (34). We found the two methods to be highly sensitive (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 (34). In 12 samples, both techniques detected MRD levels $>10^{-4}$. 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 $<10^{-4}$. Results were discordant in only two of these samples: PCR detected 2 in 10^4 and 5 in 10^4 leukemic cells, whereas the results of the flow-cytometric assays were negative; both patients remain in remission by clinical, flow-cytometric, and molecular criteria, 22 and 32 mo after remission.

We also compared the results of flow cytometry to those of reverse transcription (RT)-PCR amplification of fusion transcripts (*BCR-ABL* and *MLL-AF4*; Coustan-Smith 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 (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 levels of MRD associated with a higher risk of relapse (i.e., $>10^{-4}$) can be detected by either technique.

Potential Sources of Error

Detection of rare events by flow cytometry requires meticulously clean and precise procedures (16). False-positive results can be caused by sample contamination, dirty reagents, and imperfect cleansing of the fluidics system. False-positive results can be caused by using antibodies that react nonspecifically. We strongly recommend the use of isotype-matched nonreactive antibodies as

controls and careful titration of all antibodies. The sequence in which antibodies are added to cells and the times of incubation must be rigorously standardized, because variations in these procedures can alter the intensity of cell labeling. Variations resulting from changes between different batches of antibodies must be monitored by frequent staining of normal samples. It goes without saying that the instrument should be maintained in excellent condition, with frequent calibration and periodic servicing.

A small fraction of patients has a recurrence of acute leukemia whose cellular features are unlike those determined at diagnosis. In the majority of cases, these leukemias are unrelated to the original leukemic clone and represent secondary malignancies, which are often caused by the mutagenic effects of leukemia treatment (35,36). Clearly, secondary leukemias cannot be anticipated by currently available methods for monitoring MRD. In a proportion of cases, recurrent leukemia has genetic features that confirm its relationship to the original leukemic clone, but has the phenotype of a different lineage (lineage switch). There have been reports of leukemias morphologically and immunophenotypically characterized as ALL that relapse as AML, while retaining the karyotypic and molecular features of the original clone (37–39). Such “lineage switch” relapses may be detected early by molecular methods such as including PCR amplification of chromosomal breakpoints or antigen-receptor gene rearrangements, but not by flow cytometry.

Another cause of false-negative results during monitoring for residual leukemia is clonal evolution during and after treatment, which may cause the disappearance of one or more of the markers detected at diagnosis—a phenomenon already noted in early studies (40–46). The impact of immunophenotypic changes on MRD monitoring with multiple markers is related to the number of marker combinations that can be applied to each patient.

CLINICAL STUDIES OF MRD IN ALL BY FLOW CYTOMETRY

Correlation Between MRD and Treatment Outcome

Immunophenotyping was the first method to be productively used to study MRD (3). Several earlier studies demonstrated the potential usefulness of this approach (24,47,48). Despite promising initial results, interest in this approach was somewhat diverted by the advent of PCR in the late 1980s. Many investigators, startled by the novelty and elegance of PCR, began regarding almost any other existing laboratory technique as a relic of another era. However, when we directly compared the two methods over a decade ago (49), we emerged with the impression that flow cytometry would remain a valid, informative, and clinically applicable approach to study MRD. This impression was corroborated by the consistent improvement in antibody and fluorochrome quality and variety and by the relentless refinement of flow cytometers and analytical hardware and software.

Our findings on monitoring residual disease in children with ALL have been summarized in a recent publication (50). We prospectively studied MRD in 195 children with newly diagnosed ALL in clinical remission. Bone marrow aspirates ($n = 629$) were collected at the end of remission induction therapy and at three intervals thereafter. Detectable MRD (i.e., $\geq 0.01\%$ leukemic mononuclear cells) at each time-point was associated with a higher relapse rate ($p < 0.001$); patients with high levels of MRD at the end of the induction phase ($\geq 1\%$) or at wk 14 of continuation therapy ($\geq 0.1\%$) had a particularly poor outcome. The incidence of relapse among patients with MRD at the end of the induction phase was $68 \pm 16\%$ (SE) if they remained MRD⁺ (18 patients) through wk 14 of continuation therapy, compared with $7 \pm 7\%$ if MRD became undetectable (14 patients) ($p = 0.035$). The persistence of MRD until wk 32 was highly predictive of relapse (all four MRD⁺ patients relapsed versus two of the eight who converted to undetectable MRD status; $p = 0.021$).

Residual disease was significantly more frequent in infants and patients ≥ 10 yr of age than in children of intermediate ages ($p = 0.007$). Notably, four of six infants had $\geq 0.01\%$ leukemic cells at the end of remission induction. Among cellular features, rates of detection did not differ significantly in comparisons based on cell lineage, but there was a strong association between MRD detection and the Philadelphia chromosome: All eight cases with this abnormality had positive findings ($p < 0.001$). This contrasts with MRD positivity in 2 of 15 cases with a *TEL* gene rearrangement and 8 of 42 cases with hyperdiploid (>50 chromosomes) B-lineage ALL, both considered favorable prognostic signs (51–55).

The predictive strength of MRD remained significant even after adjusting for adverse presenting features. It also remained significant in analyses that excluded patients at very high or very low risk of relapse by St. Jude criteria (56) or that focused on patients with high risk of relapse by NCI criteria (57). Because persistence of circulating lymphoblasts after the first week of treatment identifies children with ALL at a higher risk of relapse (58–62), we also determined whether MRD studies at the end of remission induction would add to the prognostic information provided by the earlier morphologic assessment of circulating lymphoblasts. MRD findings at the end of the induction phase correlated well with treatment outcome in patients with or without circulating blasts.

Additional findings demonstrating the value of immunologic MRD monitoring in patients with ALL were reported by Farahat et al. (63), who used antibodies to TdT, CD10, and CD19 to detect MRD in six of nine patients 5–15 wk before relapse. By contrast, 43 patients who remained in continuous complete remission, with a median follow-up of 23 mo, were consistently free of MRD by flow cytometry. In a study of 53 ALL patients (37 B-lineage and 16 T-lineage ALL), Ciudad et al. (64) used three-color flow cytometry to study MRD. Patients who had a gradual increase in MRD levels showed a higher relapse rate (90% vs 22%) and shorter median relapse-free survival than those with stable or decreasing

MRD levels. The adverse predictive value of MRD was also observed when children and adults were analyzed separately.

Bone Marrow Versus Peripheral Blood for MRD Studies

Practical and ethical considerations limit the acquisition of sequential bone marrow samples from children. The use of peripheral blood rather than bone marrow may provide additional opportunities for MRD studies, but little is known about the clinical significance of studying MRD in peripheral blood. The existing studies on the subject have used PCR and produced discordant results. Brisco et al. used quantified MRD in 35 paired blood and bone marrow samples from 15 children with B-lineage ALL receiving induction therapy and found that the level of MRD in peripheral blood was approx 10-fold lower than in marrow (65). Van Rhee et al., in a study of Ph+ ALL, had similar findings in 3 of 18 patients, while in the remainder, there was no significant difference in MRD detected in blood and marrow (66). Martin et al. also found that MRD levels in marrow exceeded those in blood by a factor of 10 or more in six patients (67). However, more recently, Donovan et al. 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 (68).

We studied 90 pairs of bone marrow and peripheral blood samples. Of these, 69 were negative in both marrow and blood and 10 were positive in both. In the remaining 11 samples, leukemic cells were detected in the bone marrow but not in peripheral blood. Interestingly, all five patients with T-lineage ALL who had detectable MRD in the bone marrow had an approximately equal proportion of leukemic cells in the peripheral blood. By contrast, only 5 of the 16 patients with B-lineage ALL who had detectable MRD in the bone marrow also had detectable circulating blast cells (Coustan-Smith et al., unpublished results). Taken together, the available evidence suggests 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.

FUTURE PERSPECTIVES

The studies of MRD in childhood ALL reported to date collectively indicate that measurements of MRD provide a powerful and independent prognostic indicator of treatment outcome in children with ALL and are likely to have a consequential impact on the clinical management of these patients. The results of MRD studies during the early phases of therapy in this disease are consistent with, and add to, the predictive value of other measurements of early response to therapy, such as the presence of circulating blast cells at d 7 of therapy (59), the degree of response to prednisone (61), and the morphologic detection of blast cells in the bone marrow on d 15 and 21 (69).

It remains to be decided how MRD assays should be used to guide treatment. Based on the existing evidence, it seems reasonable to intensify therapy for those patients who have a slow early response to treatment and have detectable MRD during clinical remission. Conversely, the excellent clinical outcome of MRD-negative cases raises the possibility of using MRD assays to identify candidates for experimenting reductions in treatment intensity. However, it may be argued that studies of MRD are unlikely to substantially improve clinical strategies in a disease such as childhood ALL, in which approximately three-fourths of patients can be cured and for which several risk factors strongly predictive of outcome are already guiding therapy (70). However, known prognostic factors are not 100% predictive, and MRD studies might well complement and enhance their informative value. Moreover, oncologists may be reluctant to abandon clinical and biologic parameters, such as age, leukocyte counts, and genetic features, whose relation with treatment response has been repeatedly confirmed, even within different treatment protocols, and there are only a few informative (but not nearly as extensive) clinical studies of MRD. Therefore, at present, it seems prudent to combine MRD with clinical and biologic parameters for a comprehensive risk assignment in children with ALL.

We still do not know whether early detection of relapse and subsequent changes in therapeutic strategies will improve cure rates, but there is reason to believe that this might be the case. First, it is well established that the tumor burden and the curability of cancer are related. In ALL, for example, a large tumor mass at diagnosis as demonstrated by high leukocyte counts and high serum lactate dehydrogenase activity is an indicator of poor prognosis (56). Second, the likelihood of the emergence of drug-resistant malignant cells by mutation increases as the number of cell divisions increases and, hence, relates to the total tumor burden (71).

The use of MRD studies may benefit treatment of childhood ALL beyond risk assignment. For example, the utility of autologous transplantation could conceivably be improved by the development of effective techniques for purging the graft of leukemic cells, coupled with sensitive methods for detection of MRD. In addition, testing of new treatment approaches, such as tyrosine kinase inhibitors, cytokines, immunotoxins, adoptive T-cells, compounds interfering with oncogenic molecular aberrations, and inhibitors of angiogenic growth factors, may necessitate modifying the way in which anticancer treatments have traditionally been tested. MRD measurements may serve as surrogate end points in the clinical testing of these novel therapeutic approaches.

One has to recognize that none of the methods developed to date to study MRD is perfect and that existing techniques have advantages and disadvantages. Therefore, our approach is to combine two methods in efforts to study all patients. By using flow cytometry and PCR amplification of antigen-receptor genes simultaneously, we have been able to study 96 consecutive cases. This approach

should also prevent false-negative results because of changes in immunophenotype or predominant antigen-receptor gene clone during the course of the disease.

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