Detection of Minimal Residual Disease in Acute Leukemia: Methodologic Advances and Clinical Significance

By Dario Campana and Ching-Hon Pui

AT DIAGNOSIS, PATIENTS WITH acute leukemia may have a total of approximately $10^{12}$ malignant cells.1,2 The disease is considered to be in complete remission when fewer than 5% of the cells in bone marrow (BM) samples are morphologically identifiable blasts. However, these patients may still have as many as $10^6$ neoplastic cells. From that point until overt clinical relapse, the level of leukemic cells in the body is largely unknown, resulting in clinical management strategies that do not discriminate among patients by their levels of residual disease. Thus, patients with $10^6$ leukemic cells are treated on the same regimen as those with much lower levels or, perhaps, with no leukemia at all. Although the sensitivity of morphologic studies can be improved somewhat by cytochemical staining or by examining mononucleated cell fractions, detection of neoplastic cells comprising less than 1% of a cell population remains beyond the resolution of this method.3,4

The premise underlying studies of minimal residual disease (MRD) is that better estimates of the total body burden of leukemic cells would improve clinical management and advance cure rates. The close relation between the size of the tumor burden and the curability of acute leukemia is well established.2 In acute lymphoblastic leukemia (ALL), for example, high white blood cell counts and high serum levels of lactic dehydrogenase at diagnosis, indicative of a large tumor mass, are, in turn, poor prognostic indicators.5,6 In theory, intensive therapy delivered when a tumor burden is small should increase the likelihood of cure.7 Drug-resistant malignant cells can emerge by mutation, even in the absence of a selecting drug, and their number is likely to depend on the number of cell divisions that have occurred and, hence, on the total tumor burden.2,7,8 Thus, the larger the volume of leukemic cells, the higher the likelihood of developing multiple drug-resistant mutants. Accordingly, detection of residual leukemia at low levels, when no or only a few drug-resistant blasts have arisen, would offer unique opportunities for therapeutic intervention.

Leukemic cells can be distinguished from normal hematopoietic progenitors on the basis of morphologic and cytochemical properties, karyotypic or genetic abnormalities, antigen-receptor gene rearrangements, cell growth requirements in vitro, and immunophenotype. Combinations of these characteristics have been exploited to detect small numbers of neoplastic blasts among normal cells. The following sections review recent progress in the development of MRD assays, with emphasis on their potential for clinical application.

DETECTION OF CELLS WITH KARYOTYPIC ABNORMALITIES

Conventional cytogenetic techniques. Conventional karyotyping, based on chromosomal abnormalities that were noted at diagnosis, has been used to monitor residual disease.9-12 The chief advantage of this approach is that it permits unambiguous identification of leukemic cells. In early studies of patients with ALL and acute myeloid leukemia (AML), disappearance of the abnormal karyotype generally coincided with the induction of clinical (morphologic) remission. Hart et al10 reported this observation in 9 patients with AML and 1 patient with ALL. Each of the 8 patients who relapsed had the same karyotypic abnormality that was found at diagnosis. Testa et al11 also failed to detect chromosomally abnormal cells in remission BMs of patients with AML, although single abnormal cells of clonal origin could occasionally be observed. More recently, however, Freireich et al,12 reported that abnormal metaphases, identical to those found at diagnosis, were detected in 20 of 71 patients with AML, in morphologic remission. All 20 patients relapsed within the next 78 weeks, supporting the reliability of positive karyotypic analysis as a predictor of eventual relapse. However, 25 of the 51 patients with negative findings also relapsed, indicating that failure to detect abnormal clones does not guarantee durable remissions.

Metaphase analysis by conventional banding techniques is a laborious procedure. Its success depends on the number of metaphases that can be examined and on the proliferative rate of leukemic cells, which may vary from case to case.9,13 Thus, there has been considerable effort to develop techniques that would facilitate metaphase screening and allow karyotypic analysis of nondividing cells.

In situ hybridization. Fluorescence in situ hybridization (FISH) techniques rely on chromosome-specific and genespecific DNA probes to identify numeric and structural chromosomal abnormalities.14,15 They can be combined with morphologic analysis or immunohistochemical assays to improve the accuracy of results.16,17 The main advantage of this approach is that it provides interpretable information with use of interphase (nondividing) cells, increasing the chances of identifying chromosomal abnormalities among cells with a low proliferative rate.

Using FISH with an X-chromosome-specific probe, Anastasi et al18 detected trisomy X in 1.6% of the interphase cells in a BM sample from a patient with trisomy X-positive ALL in morphologic and cytogenetic remission. They also detected this abnormality in a subsequent BM sample ob-

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tained at relapse. The same group followed two children with ALL and trisomy 17 using an α-satellite DNA probe specific for chromosome 17. In the end-of-therapy sample from one patient, in whom morphologic results were uncertain, approximately 10% of the cells apparently had trisomy 17. Overt leukemia was diagnosed in this patient 2.5 months later. In a similar sample collected from the second patient, all cells appeared to be diploid. This child had been in complete remission for 12 months at the time of publication of the written report. The sensitivity of FISH is shown well by research of Heerema et al, who used probes to chromosomes X, 10, 17, and 18 to monitor early remission in hyperdiploid ALL. The mean number of leukemic cells observed in three normal BM samples was 8 of 2,000. One month after diagnosis, trisomic and tetrasomic interphases were significantly increased over control values in three of the seven patients studied, and pentasomy and hexasomy, not found in control samples, were observed in five of the seven cases studied.

Nyland et al used FISH to detect numerical chromosomal abnormalities in interphase and metaphase cells, as well as specific translocations in metaphase cells, in patients with various hematologic malignancies. Of seven evaluable patients with AML, three had cells bearing abnormal karyotypes in morphologic remission BM samples, two of whom subsequently relapsed. By contrast, none of the remaining patients with “FISH-negative” remission samples has had a recurrence. FISH analysis of remission in ALL appeared to be less informative. Although no abnormal karyotypes were detected in remission BM samples of five patients, two subsequently relapsed.

Because it is limited by the presence of aneuploid (but not leukemic) cells and by certain inherent technical artifacts, the sensitivity of MRD analysis by FISH approaches only 1%. At least one attempt has been made to increase this level of sensitivity by applying the procedure to fluorescence-activated cell sorter (FACS)-sorted cells (eg, CD34+) and relying on bromodeoxyuridine labeling to simultaneously assess the cells’ proliferative activity. In a study of 13 patients with AML in complete remission, Drach et al investigated the presence of numerical chromosomal abnormalities (monosomy 7 and trisomy 8) on preselected CD34+ cells from BM. Of the 5 patients with residual disease, 4 relapsed in a median time of 16.5 weeks. By contrast, 6 of 7 patients without detectable MRD remained in complete remission (median follow-up, 37.5 weeks).

Techniques to detect structural chromosomal abnormalities in interphase cells by in situ hybridization with locus-specific probes have also been developed. For example, probes directed against the BCR and ABL genes, each labeled with a different fluorochrome, can be used to identify the t(9;22) abnormality. In cells bearing the translocation, one set of probes colocalizes in one region of the interphase nucleus. However, the sensitivity of this approach may be limited because, in about 5% of normal lymphocytes, artificial colocalization may be observed. Finally, RNA in situ hybridization has been used by one group of investigators to detect genes whose expression was putatively amplified in leukemic cells. However, the validity of this approach remains in question.

Flow cytometry. Some groups have proposed the use of flow cytometry as a means to detect chromosomal abnormalities with a higher accuracy than conventional banding techniques. One approach entails two-color analysis with chromomycin A3 (which labels GC base pairs) and Hoechst 33258 (which labels AT base pairs), a procedure that resolves all chromosomes but numbers 9 to 12. However, this approach is not more sensitive than conventional banding techniques.

A second method relies on the identification of aneuploidy by single-laser cytometry in cells labeled with DNA-binding fluorochromes, such as propidium iodide and 7-actinomycin D. This simple technique affords the possibility of monitoring MRD in most hyperdiploid and hypodiploid cases. For example, Pantazis et al monitored the course of a patient with M7 AML reporting that the disappearance of aneuploid peaks by flow cytometry coincided with attainment of morphologic remission, and Redner et al detected early relapse in a patient with near-haploid ALL who was in clinical remission. Walle and Niendorreyer have reported improvement in the sensitivity of detection of aneuploidy after separation of cells by density gradient procedures. Negative results were followed by durable remissions in three patients with ALL, whereas positive findings preceded overt relapse in two other patients. A more selective examination of progenitor cells is achieved by combining DNA ploidy and cell marker studies, as shown by Tsubosawa et al, who analyzed the DNA content of BM CD10+ cells in a patient with hypodiploid B-lineage ALL. Sequential examination of so-called remission BM samples showed the persistence of leukemic cells that preceded overt relapse.

DETECTION OF GENETIC ABNORMALITIES BY THE POLYMERASE CHAIN REACTION (PCR)

Molecular targets. The chromosomal alterations that are candidates for amplification by the PCR are listed in Table 1. Whether genomic DNA or cDNA obtained from RNA after a reverse transcription-PCR (RT-PCR) step is used in this procedure depends on the molecular target. If one can design PCR primers matching conserved regions that flank the translocation junction and are separated by a relatively short stretch of DNA (no more than a few hundred base pairs), then genomic DNA is selected. If, on the other hand, the flanking primers are separated by longer stretches of DNA involving both introns and exons, a cDNA fusion transcript, from which intervening sequences have been spliced, is preferable. The sensitivity of PCR for detecting karyotypic abnormalities is extremely high. Experiments with artificial mixtures of leukemic and normal cells or DNA have consistently shown detection of a single leukemic cell among 107 to 108 normal cells.

In approximately 3% to 5% of cases of childhood ALL, leukemic cells have a reciprocal translocation between chromosomes 9q34 and 22q11 (Philadelphia [Ph]-chromosome), which disrupt the ABL and BCR genes, respectively (Fig 1). The incidence of Ph-positive ALL is much higher in adults, eg, 51% in a retrospective study of 314 common ALL cases, a proportion recently confirmed by the same investigators in a prospective study.
notypic subtypes are considered, approximately one third of adult patients with ALL have BCR-ABL recombination.\textsuperscript{6,42} Cytogenetically, the Ph-chromosomes found in ALL are indistinguishable from those found in chronic myelogenous leukemia (CML), but they differ at the molecular level (Fig. 1).\textsuperscript{1,23}\textsuperscript{44} Whereas the breakpoints on chromosome 22 in CML virtually always map to the major breakpoint cluster region of the BCR gene, most Ph-positive ALL patients show a translocation to the minor breakpoint cluster region of the same gene.\textsuperscript{38,39,43,44} In both cases, the breakpoints on chromosome 9 may be scattered along a large region, resulting in DNA sequences that are too variable for PCR analysis of DNA.\textsuperscript{37}\textsuperscript{44} However, the transcribed BCR-ABL messenger RNA is consistent and can be used as a template for RT of complementary DNA, which in turn can be amplified with use of suitable BCR- and ABL-matching primers.\textsuperscript{25,37}

The other two major translocations in childhood ALL, the t(1;19)(q23;p13.3) and the t(4;11)(q21;q23), can also be studied by RT-PCR.\textsuperscript{45-48} All cases of surface Ig-positive B-ALL and Burkitt’s lymphoma have translocations involving the MYC proto-oncogene on the 8q24 region and the Ig receptors-a gene, most commonly the heavy-chain gene at 14q32.\textsuperscript{9} However, the transcribed BCR-ABL messenger RNA is consistent and can be used as a template for RT of complementary DNA, which in turn can be amplified with use of suitable BCR- and ABL-matching primers.\textsuperscript{25,37}

Table 1. Genotypic Abnormalities That Can Be Used as Targets for Molecular Detection of MRD

<table>
<thead>
<tr>
<th>Disease</th>
<th>Chromosomal Abnormality</th>
<th>Molecular Target</th>
<th>Frequency (%)*</th>
<th>Adults</th>
<th>Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>t(1;19)(q23;p13.3)</td>
<td>BCR-ABL (RNA)</td>
<td>25-40</td>
<td>4-6</td>
<td></td>
</tr>
<tr>
<td>B-lineage</td>
<td>t(4;11)(q21;q23)</td>
<td>E2A-PBX1 (RNA)</td>
<td>2-3</td>
<td>5-6</td>
<td></td>
</tr>
<tr>
<td>T-lineage</td>
<td>t(11;19)(q23;p13)</td>
<td>MLL-AF4 (RNA)</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>T-lineage</td>
<td>t(8;14)(q24;q32.3)</td>
<td>IL3-IGH (DNA)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>T-lineage</td>
<td>t(8;14)(q22;q12)</td>
<td>MLL-ENL (RNA)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>t(8;21)(q22;q22)</td>
<td>MLL-AF9 (RNA)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>t(9;11)(p21-22;q23)</td>
<td>E2A-HLF (RNA)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>T-lineage</td>
<td>t(11;14)(p13q11)</td>
<td>MYC-IGH (DNA)</td>
<td>4-5</td>
<td>1-2</td>
<td></td>
</tr>
</tbody>
</table>

* The frequency within B-lineage, T-lineage, or AML disease group.

Acute promyelocytic leukemia (APL; M3 AML).\textsuperscript{56-58} The breakpoints of this translocation affect the retinoic acid receptor-\(\alpha\) gene (RARA) on chromosome 17 and a transcription factor, PML, on chromosome 15.\textsuperscript{56-58} PCR protocols to detect the AML1-ETO fusion transcript in the t(8;21)(q22;q22), CBFB-MYH11 in inv(16)(p13q22), DEK-CAN in t(6;9) (p23;q34), and MLL-AF9 in t(9;11)(p21-22;q23) have also been reported.\textsuperscript{59-63} N-RAS mutations, found in approximately 25% of AML\textsuperscript{64} and 20% of adult ALL cases,\textsuperscript{65} could potentially be used as a target for PCR studies of MRD; however, the mutations often differ at the time of relapse, so that one might obtain an unacceptably high number of false-negative results.\textsuperscript{66}

False-positive PCR results can derive from contaminating traces of DNA, particularly when one is amplifying translocation breakpoints using the same set of primers and probes in different cases.\textsuperscript{67} These errors can be avoided by careful manipulation and processing of samples, and the use of multiple controls. In the case of RNA amplification by RT-PCR, it is possible that some of the “clonogenic” malignant cells with the chromosomal translocation do not express the corresponding RNA, as has been suggested for early leukemic progenitor cells in CML.\textsuperscript{68} This event may cause failure to detect MRD.

Clinical studies. Among the leukemic lymphoblast-specific fusion transcripts that have recently become targets of PCR analysis, only those associated with the Ph chromosome have been systematically applied to the study of MRD. Analysis of 17 BM and 12 peripheral blood (PB) samples from 12 Ph\textsuperscript{+} ALL cases in complete remission for 3 to 14 months failed to detect BCR-ABL fusion transcripts in 9 samples, including BM samples of 3 patients.\textsuperscript{69} Similarly, in another study, 2 of 10 Ph\textsuperscript{+} ALL cases in cytogenetic remission were negative by PCR after chemotherapy but before BM transplantation (BMT).\textsuperscript{70} That intensive chemotherapy can reduce
the majority of ALL cases map to the minor breakpoint cluster region, regions are indicated by double pointed arrows. The breakpoints produce a transcript of 7.0 kb encoding a protein of 190 kD. In most cases of Ph+ ALL, the breakpoints map to the major breakpoint cluster region of 5.8 kb, located downstream from minor breakpoint cluster region. This rearrangement produces 8.5-kb transcripts encoding a protein of 210 kD. Single-pointed horizontal arrows indicate the position of the primers used for RT-PCR.

BCR-ABL Transcripts

Fig 1. BCR/ABL recombination in Ph+ leukemias. The breakpoints regions are indicated by double pointed arrows. The breakpoints in the majority of ALL cases map to the minor breakpoint cluster region, located within the BCR first intron of approximately 68 kb. This produces a transcript of 7.0 kb encoding a protein of 190 kD. In most cases of CML, the breakpoints map to the major breakpoint cluster region of 5.8 kb, located downstream from minor breakpoint cluster region. This rearrangement produces 8.5-kb transcripts encoding a protein of 210 kD. Single-pointed horizontal arrows indicate the position of the primers used for RT-PCR.

The RT-PCR assay has proved a useful method for detecting MRD in APL with the PML-RARA fusion gene. In APL, chemotherapy with or without all-trans-retinoic acid or BMT generally leads to rapid loss of PCR positivity, with subsequent findings correlating closely with treatment outcome. In the study of Lo Coco et al, 11 of the 13 patients with positive PCR results 4 months after induction of clinical remission relapsed 1 to 4 months later, whereas all 22 patients with negative results at 4 months remained disease-free for 3 months to 5 years later. These investigators subsequently studied nine APL cases in long-term remission for 4 to 12 years after chemotherapy or BMT, none of which had detectable PML-RARA fusion transcripts in their BM. 73

The predictive value of PCR reactivity in APL was confirmed by other groups of investigators. 74-76 These studies showed that, although all-trans-retinoic acid treatment could induce remission in a high proportion of patients with APL, it rarely led to PCR negativity when used alone. 74,75 Additional chemotherapy or BMT was necessary to eliminate PCR evidence of PML-RARA transcripts and to prolong remissions. Thus, PCR negativity should be considered a goal in the clinical management of this disease. In this regard, serial negative results were correlated with prolonged disease-free survival, whereas one or more positive tests after treatment were associated with subsequent relapse. 75

The clinical value of PCR monitoring in acute myeloblastic leukemia (French-American-British classification M2) cases with the t(8;21) using the AML1-ETO fusion gene is less certain. In four separate studies, the fusion transcript was detected in BM or even PB samples of five patients who had completed all treatment and remained in remission for 5 to 8 years. 77-80 Whether expression of the chimeric protein does not itself constitute a transformed phenotype or whether the malignant cell proliferation in these patients was suppressed by host immunity is unclear. However, this finding raises questions about the validity of detection of fusion gene detection as a predictor of clinical outcome in some leukemias. Indeed, PCR results are often positive in CML patients after BMT, but they do not consistently indicate a poor prognosis. 69,70,81-84

DETECTION OF MINIMAL RESIDUAL DISEASE

Rationale and technical aspects. The antigen-receptor genes comprise several discontinuous germline segments (ie, V, variable; D, diversity; and J, joining) that undergo rearrangement in lymphoid cells. The uniqueness of the rearrangement derives from the use of one of the many gene segments available from each pool of the V, D, and J gene segments, and from the variability at the recombination site caused by deletion of germline nucleotides and insertion of non-templated nucleotides ("N regions"), a reaction mediated by terminal deoxynucleotidyl transferase (TdT). 85

Because such rearrangements are clonal (unique for an individual cell and its progeny), analysis of Ig and T-cell receptor (TCR) gene configurations can be used to track the persistence of malignant clones whose rearrangements were determined at diagnosis. In B-lineage ALL, greater than 95%, 54%, 55%, and 33% of cases have rearranged IgH, TCRβ, TCRγ, and TCRβ genes, respectively. The same genes are rearranged in 14%, 68%, 91%, and 89% of T-ALL cases, respectively. 86 Thus, both lineage-associated and "lineage-inappropriate" rearrangements can be used as a residual disease marker in ALL.

The sensitivity of conventional DNA analysis techniques (eg, Southern blotting of nonamplified DNA) is limited (1% to 5%; see Table 2), similar to that of morphologic techniques. 83,86 This low sensitivity may be improved by focusing the DNA analysis on selected subsets of cells, such as mononuclear cells isolated by density gradients or immunologically defined cells purified by cell sorting with immunomagnetic particles. 87

Rearrangement of the various antigen-receptor regions is accompanied by deletion of intervening sequences, such that
the gene segments are repositioned closer to each other in the rearranged gene sequence (Fig 2). Thus, enzymatic gene amplification using PCR can detect rearranged genes with greater sensitivity (1 target cell in 10^1 to 10^5 cells) than Southern blot analysis of nonamplified DNA. The sensitivity of PCR for antigen-receptor gene rearrangements varies according to the uniqueness of the leukemia-specific regions of the genes. Short N regions may lead to a low specificity of the genes and hybridization with clonospecific probes, the detection level was in 10^6 or fewer normal cells in 42 of 88 cases. Similarly, in 71 cases studied by PCR amplification of TCRγ genes and hybridization with clonospecific probes, the detection level was 1 leukemic cell in 10^6 or fewer normal cells in 33 cases. A sensitivity of 1 in 10^5 was achieved in 29 cases, whereas 1 in 10^6 was attainable in only 9 of the 71 cases.

Table 2. Methods for Detecting Clonality of Ig and TCR Gene Rearrangements

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern blotting</td>
<td>1-5</td>
<td>86, 87</td>
</tr>
<tr>
<td>Immunoselection + Southern blotting</td>
<td>0.05</td>
<td>88</td>
</tr>
<tr>
<td>PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene fingerprinting</td>
<td>0.1-0.01</td>
<td>92, 103, 104</td>
</tr>
<tr>
<td>Probes prepared from restriction</td>
<td>0.1-0.001</td>
<td>42, 101, 111, 112, 125, 129, 131</td>
</tr>
<tr>
<td>enzyme digestion of PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amplified diagnostic DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAse protection assay</td>
<td>0.001</td>
<td>113</td>
</tr>
<tr>
<td>Probes or primers synthesized after</td>
<td>0.1-0.001</td>
<td>89, 98, 99, 105-110, 126, 127, 130</td>
</tr>
<tr>
<td>sequencing PCR-amplified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>diagnostic DNA</td>
<td></td>
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</tr>
</tbody>
</table>

* Percentage of detectable clonal DNA or cells.

One can design primers specific for individual V and J regions, or consensus primers for conserved regions. For example, the approximately 100 VH genes can be grouped into seven families with homologous sequences. The most conserved regions are known as framework regions, whereas the regions that encode the antigen-binding site of the Ig heavy chains and undergo somatic mutation during the immune-response ("hypervariable segments") are termed complementarity-determining regions (CDRs). Two CDRs (CDR1 and CDR2) are encoded by the VH gene region. By contrast, the CDR3 region comprises the 3' end of VH, all of D, and the 5' end of JH. It also contains N nucleotides and is assembled during the recombination process. This region is different in each lymphoid clone. To amplify rearranged IgH genes, one can use a consensus JH primer with a panel of VH primers specific to VH families or with a single VH primer matching a conserved region of most VH genes. With the former approach, Ig gene rearrangements can be detected in greater than 90% of B-lineage ALL cases at diagnosis; with the latter, a success rate of approximately 75% has been reported. A combination of the two methods has been reported to improve the detection rate so that only a small proportion of cases (those with incomplete rearrangements) escape amplification. IgH gene rearrangements are usually incomplete in T-ALL.

TCRα and TCRβ genes have a large number of functional V and J segments, whereas TCRγ and TCRδ contain only a few. Thus, although the potential for combinatorial diversity is greater for TCRα and TCRβ than for TCRγ and TCRδ, the latter's diversity is substantial, deriving primarily from the diversity in the junctional region, which consists of D-regions (only in TCRβ and TCRδ), N-regions of randomly inserted nucleotides, P-regions (nucleotides forming a palindromic sequence with the juxtaposed nucleotides), and deletion of nucleotides from the ends of the gene segments involved in the junction. Efforts to detect rearranged TCRγ genes using consensus primers to the Vγ1 family and to the Jγ1 and Jγ2 segments are successful in approximately 90%

**Wild-type TCRδ Locus**

![TCRδ gene recombination in ALL. Two main types of rearrangement are found in ALL. The Vδ1-D-Jδ1 recombination is predominant in T-ALL, whereas the Vδ2-D-Jδ3 is usually found in B-lineage ALL. Arrows indicate the position of the primers that can be used to amplify the recombination sites by PCR.](image-url)
of cases.\textsuperscript{98,99} Rearrangements of TCR\textgreek{y} and TCR\textgreek{d} genes are found not only in T-ALL, but also in B-lineage leukemias; however, incomplete TCR\textgreek{d} rearrangements with short junctional regions (ie, V\textgreek{6}2-D\textgreek{6}3 and D\textgreek{6}3-D\textgreek{6}3) are relatively common in B-lineage ALL, in contrast to the dominant V\textgreek{6}1-D\textgreek{6}1 rearrangements in T-ALL (Fig 2).\textsuperscript{100,102}

In contrast to findings with abnormal gene configurations caused by chromosomal translocations, the appearance of a PCR-amplified signal from antigen-receptor genes cannot be taken as evidence for MRD until the signal is distinguished from the background originating from normal lymphoid cells. These can be accomplished as follows. First, clonal IgH rearrangements amplified with single VH family-specific primers (selected from the reactivity of the diagnostic DNA) can be identified on the basis of size and signal intensity after separation by high-resolution gel electrophoresis.\textsuperscript{92,94,103,106} The electrophoretic profiles or "fingerprints" obtained with remission samples are compared with the diagnostic DNA for the presence of similar-sized dominant bands. Polyclonal background levels vary but usually limit the sensitivity of this approach to the detection of 1 leukemic cell among 1,000 normal cells.\textsuperscript{92,93} Second, clonally rearranged Ig and TCR genes can be distinguished by analyzing the junctional regions of TCR\textgreek{y} or TCR\textgreek{d} genes or the CDR3 regions of the IgH genes. After sequencing, PCR primers and/or clonospecific probes are synthesized for each patient.\textsuperscript{98,99,105-110} Similar to PCR amplification of translocation breakpoints, leukemia-specific primers yield PCR-amplified signals only in the presence of the malignant clone. These can be detected in ethidium bromide-stained gels. When clonospecific probes are used instead, these are labeled with radioisotopes to visualize their hybridization with DNA from leukemic cells amplified with consensus primers. Alternatively, Hansen-Hagge et al\textsuperscript{111} applied a method in which clonospecific probes are generated by primer extension and restriction-enzyme digestion using the FokI for cases with V\textgreek{6}1-D\textgreek{6}1-J\textgreek{6}1; no digestion is necessary in cases with V\textgreek{6}2-D\textgreek{6}3 recombination. With a conceptually analogous approach, Nizet et al\textsuperscript{112} prepared leukemia-specific probes by amplifying VDJ regions of IgH genes from diagnostic material, purified the rearranged bands by high-pressure liquid chromatography, and digested them with SacI. Finally, Veelken et al\textsuperscript{113} described a method for this purpose, based on the amplification of rearranged TCR\textgreek{y} genes with consensus V\textgreek{y} and J\textgreek{y} primers, one of which incorporates the RNA T7 polymerase promoter. A specific antisense RNA probe, synthesized from the diagnostic rearrangement, is used in RNase protection assays directed against sense RNA generated from the test samples amplified with the T7 promoter on the opposite primer.

The use of these approaches to detect MRD by PCR does not always guarantee specificity. For example, false-positive signals can originate from nonspecific hybridization of clonospecific probes to amplified DNA from normal lymphocytes, a pitfall that may be controlled by size discrimination of the amplified DNA.\textsuperscript{114} Another limitation of PCR applied to the study of Ig and TCR gene rearrangements is that target loci may be deleted, and additional rearrangements may become dominant ("clonal evolution") during the course of the disease.\textsuperscript{115-121} A further complication arises from differences in the pattern of IgH rearrangements observed in the same patient at the time of diagnosis in PB and BM.\textsuperscript{122} In the case of IgH genes, the leukemic cell populations may show differences in the CDR3 region (the extremely diverse region whose sequence is used as a clonal marker), which is frequently disrupted by continuous VH gene rearrangements.\textsuperscript{91,116,117} By Southern blot analysis, investigators have indeed found changes in the pattern of rearrangement at presentation and relapse for both IgH and TCR genes. Beishuizen et al\textsuperscript{119} detected biclonal or oligoclonal IgH rearrangements at diagnosis in 8 of 30 cases of B-lineage ALL. They also noted differences in the rearrangement patterns of 20 of these cases at the time of relapse, and in 5 of 10 cases of T-ALL also studied at relapse. The investigators observed a correlation between shifts in antigen-receptor gene rearrangement pattern and the duration of remission. Nevertheless, in greater than 75% of cases in this series, at least one major rearranged IgH, TCR\textgreek{y}, or TCR\textgreek{d} allele remained stable at relapse. Oligoclonality and clonal evolution in IgH genes are often caused by disruption in the V-N-D portion, whereas the D-N-J sequence is left unmodified. Rovera et al\textsuperscript{120} studied the IgH gene by PCR at diagnosis and relapse in 12 cases of ALL and detected clonal evolution in 4, which they attributed to VH gene replacement in 3 cases and to a new rearrangement with loss of the original alteration in the remaining case. Steward et al\textsuperscript{121} identified changes in the pattern of PCR amplification between presentation and relapse in 12 of 39 patients with B-lineage ALL studied for IgH gene rearrangements. In 9 of the 12 cases, there were subclones or rearrangements representing partial or complete configurations observed at diagnosis. Changes in V\textgreek{6}2-D\textgreek{6}3 rearrangements, the incomplete rearrangements of the TCR\textgreek{d} gene most often found in B-lineage ALL, were observed in 6 of 24 cases studied (original configurations were retained in only 1 of these cases). Thus, to avoid false-negative results when using IgH-PCR to detect MRD, it is advisable to select probes matching the D-N-J junctions of all the IgH rearrangements found at diagnosis and to investigate more than one gene whenever possible.

**Quantitative analysis of MRD by PCR.** One of the aims of MRD investigations is to estimate the amount of residual tumor rather than simply establish its presence. Although immunologic methods, in situ hybridization with interphase cells, and ploidy studies by flow cytometry allow precise estimates of the proportion of abnormal cells within a given population, PCR has shown much less potential as a quantifying tool. First, the amplification of target sequences does not proceed indefinitely and often reaches a plateau. Second, annealing of primers to target sequences present at low frequency may occur at different times in different tubes during the early phases of amplification. Because PCR amplification is exponential (at least in its initial cycles), small tube-to-tube variations may greatly affect the yield of the final product. Thus, there is frequently a lack of correlation between the amount of PCR product and the initial amount of target molecules.\textsuperscript{99,123,124} When mRNA is the target molecule, as is the case in many chromosomal abnormalities, two additional potential pitfalls may hamper accurate quantitation of MRD.
RNA may have been degraded to various degrees, and the efficiency of its initial conversion to cDNA by RT may be quite variable.123

Many investigators use serial dilutions of diagnostic DNA or RNA with normal material derived from BM or PB leukocytes to help determine the amount of putative leukemic cells in remission BM samples.98-106,113,124 The intensity of the signal in the remission BM sample is compared with that from, for example, 100% diagnostic material and 100% normal control, in addition to serial 10-fold dilutions of leukemic DNA/RNA ranging from 10% to 0.0001%. The tube-to-tube variability and the nonlinearity of the PCR reaction affect the accuracy of this approach.99,123,124 In the dilution experiments, the signals obtained from different concentrations of leukemic material (eg, two-log difference) often look remarkably similar.

To improve MRD measurements, PCR amplification of the N-RAS gene has been used in conjunction with that of IgH genes.99,124 In two-step dilution experiments, Brisco et al125 first performed 10-fold dilutions to determine the dilution point at which PCR became negative, followed by serial twofold dilutions with five replicates each in the region of the highest dilution showing amplification and the lowest not showing amplification. The numbers of amplifiable leukemic and N-RAS genes per nanogram of BM DNA were then determined, and the level of residual disease was calculated from their ratio, assuming one IgH and two N-RAS genes in each leukemic cell.

Yamada et al126 established the percentage of leukemia-derived CDR3 sequences among normal B-cell-derived sequences by screening bacteriophage libraries of CDR3 sequences, prepared after PCR amplification of rearranged IgH genes. They used hybridization with JH consensus probes (to detect any B-cell-derived sequences in the plaques) and with leukemia-specific probes. Comparison of the number of plaques hybridizing with each probe allows an estimate of the percentage of B-lineage ALL cells in the population of B-lymphoid cells in the BM, the amount of which can, in turn, be easily calculated by flow cytometry. This approach has been repeatedly used by these investigators in retrospective studies,107,126,127 although others have deemed it too cumbersome for clinical application.99

Several competitive PCR assays have been applied to quantify BCR-ABL signals.32-34,128 A competitive PCR assay has also been used to measure the amount of leukemia-derived TCRγ and TCRδ signals.99 In this technique, a known amount of an "internal standard," represented by DNA from monoclonal cells whose rearrangements involve the same regions that are rearranged in the leukemic clone under investigation yet contain distinct DNA sequences, is added to the PCR mixture. Serial dilutions of leukemic blasts and normal cells are prepared, and dot blots of PCR products are hybridized with both a leukemia-specific probe and a probe for the internal standard. The ratio of the two sets of signals is calculated and plotted as a function of the precalculated number of blasts. This calibration curve is then used to calculate the number of leukemic cells present in remission samples.

Clinical studies. Attempts to relate PCR findings in ALL to the clinical outcome of therapy have yielded conflicting results (Table 3). Although in some series there appears to be good agreement between the detection of MRD and relapse,99,106,109-129,130 in others, detectable MRD has persisted at least for the first 1.5 to 2 years of therapy in most patients who have remained in continuous complete remission.101,107,131 Thus, a single positive PCR result early in remission does not indicate impending relapse. Sequential studies may be more predictive of clinical outcome. That is, the persistence or steady increase of positive assay findings has generally preceded clinical relapse, whereas a continuous decrease in levels of PCR reactivity with eventually negative results has been associated with a favorable outcome.99,123,131

Bartram and colleagues132 recently updated their MRD studies in ALL in which they used clone-specific TCRδ probes after PCR amplification. Of 71 patients investigated (58 children and 13 adults), 55 were in continuous complete remission after remission induction chemotherapy. Among the 55 patients in continuous remission, 16 of the 20 remission samples studied 1 to 6 months from diagnosis were positive by PCR, in contrast to 12 of the 35 samples studied 7 to 24 months from diagnosis and to 1 of 37 samples studied at greater than 24 months. Interestingly, the single patient who was PCR-positive while in morphologic remission greater than 24 months from diagnosis relapsed 8 months after the positive finding. In the other 16 patients with known relapse at the time of retrospective PCR investigation, 12 had persistent disease by PCR 6 to 12 months before clinical relapse, whereas in 3 patients MRD was detected only 3 to 6 weeks before relapse. In 1 patient, PCR failed to detect impending relapse caused by secondary TCR rearrangements. In another study by Potter et al,133 clinical relapse developed in two patients more than 8 years after positive posttreatment PCR tests, suggesting that remnants of the leukemic clone may be able to persist in a quiescent state for years before giving rise to overt disease. Ito et al127 specifically investigated the predictive value of assessing MRD at the end of chemotherapy in children with ALL.
the 15 patients who were in complete remission (follow-up range, 41 to 98 months), 13 had no detectable MRD, whereas in 1 the PCR signals corresponded to 1 leukemic cell in $10^9$ to $10^{10}$. In the remaining child, positive signals were detected, but sequence analysis indicated that they were not derived from leukemic cells, showing the potential for false-positive results inherent in this methodology. Of 9 patients who relapsed after completion of therapy, 8 lacked detectable leukemic cells by PCR, and 1 had positive findings. This patient's relapse was the earliest to occur in this series (2 months after cessation of therapy). Thus, the absence of MRD during treatment or at the end of therapy by PCR analysis does not ensure a durable remission.\textsuperscript{42,127,129}

Oncologists have long recognized that rapid reduction of the leukemic clone during early induction treatment is correlated with longer leukemia-free survival.\textsuperscript{132-134} This clinical observation was corroborated by recent studies showing that patients with higher levels of residual cells by PCR analysis in the immediate postinduction period relapsed earlier than the comparison group.\textsuperscript{89,93,126} In a study by Wasserman et al,\textsuperscript{126} the long-term relapse-free survival rate was 32.3% ± 11.6% for patients with high levels of residual disease (≥0.6% leukemic cells in BM), compared with 62.6% ± 10.7% for those with lower levels. Similarly, Brisco et al\textsuperscript{135} found a much higher relapse rate among patients with high levels of MRD (8 of 9), compared with those with intermediate levels (12 of 26) or no detectable MRD (29 of 117). Using more quantitative PCR methods, the same investigators extended the previous findings by showing bad, intermediate, and good outcomes for patients with high (≥$10^{-3}$), intermediate ($10^{-3}$ to $2 \times 10^{-5}$), or low ($<2 \times 10^{-5}$) levels of residual disease after intensive chemotherapy.\textsuperscript{89} Thus, patients with any amount of residual disease at the end of induction therapy should receive intensification therapy, and those with high levels of disease could become candidates for more aggressive experimental therapies. Although the studies by Wasserman et al and Brisco et al underscore the importance of MRD detection early during therapy, they differ in several conclusions, possibly because of the different treatment regimens used. In the Wasserman series, for example, patients with higher levels of MRD appeared to have increased chances of both hematologic and extramedullary relapses, whereas, in the Brisco series, only hematologic relapses were predicted by the detection of MRD at the end of remission induction. Moreover, whereas the former study predicted only relapses occurring during treatment, the latter extended to relapses off-therapy.

**CELL CULTURE TECHNIQUES**

*Technical considerations.* The rationale underlying the use of tissue culture methods to detect residual disease is that, under certain conditions, leukemic cells grow preferentially or exclusively, whereas the growth of the more numerous normal progenitors is inhibited. However, these culture conditions seem to rely on poorly defined components, such as supernatants obtained from phytohemagglutinin (PHA)-stimulated lymphocytes, human plasma, and animal sera. For example, in experiments reported by Uckun et al,\textsuperscript{135} only 3 of 17 plasma batches yielded successful cultures of leukemic lymphoblasts. In other experiments reported by Estrov et al,\textsuperscript{136} a "good" feeder cell donor was essential in establishing an optimal assay for colony growth in ALL. Another potential complication is the low numbers of leukemic colony-forming cells (ie, 0.01% to 1%) that can be detected with these assays,\textsuperscript{135-138} which could be caused by failure of the culture conditions to fulfill the survival requirements of the leukemic cells.\textsuperscript{139} Conversely, false-positive results may originate from normal cells that are able to grow in the milieu that favors leukemic growth. Additional characterization of each individual colony, such as karyotyping, antigen-receptor gene analysis, or immunophenotyping, is likely to be necessary to firmly establish the clonal origin of the colonies.\textsuperscript{140} For these reasons, it may be difficult to standardize cell culture methods to a point at which clinically useful cutoff levels of residual disease can be established and applied in prospective studies.

A modified colony assay to detect minimal residual leukemia was recently introduced by Estrov et al,\textsuperscript{141} who examined the plucked colonies by PCR. This approach should have an advantage over PCR performed on noncultured material, permitting amplification of nucleic acid material derived from proliferating leukemic cells. Moreover, clonally derived PCR products would ensure the leukemic derivation of the colonies.

**Clinical studies.** Estrov et al\textsuperscript{142} used 10% fetal calf serum, 10% PHA-leukocyte-conditioned medium, and PB leukocytes depleted of adherent cells to stimulate ALL colony formation. With this assay, they identified residual disease in 6 of 13 patients with ALL in morphologic remission, 4 of whom subsequently relapsed; each of 7 patients with negative results remained in remission, with a follow-up of 16 to 30 months.

Uckun et al\textsuperscript{143} used cell sorting and colony assays to study MRD in ALL. The critical additives to the culture medium included 2-mercaptoethanol, 15% to 30% fetal calf serum, 15% to 30% platelet-rich human plasma, 10% PHA-leucocyte-conditioned medium and/or low molecular weight B-cell growth factor and/or interleukin-2. In a series of 11 evaluable patients with B-lineage ALL undergoing autologous BMT, all 5 with greater than 0.0035% colony-forming cells relapsed, whereas 3 of the 6 patients with an equal or lower number of clonogenic cells were in remission with over 3.5 years of follow-up. In another series of 10 evaluable patients with T-ALL undergoing BMT, all 6 with greater than 0.0086% clonogenic cells in the BM (studied before purging with 4-hydro-peroxy-cyclophosphamide and immuno-toxins) relapsed soon after transplant, whereas 2 of the 4 patients with a lower frequency of colony-forming cells remained in complete remission.\textsuperscript{144} In this study, the numbers of colony-forming cells remaining after purging did not correlate with outcome. By contrast, in a series of 43 cases of AML and 15 cases of ALL undergoing autologous BMT studied by Miller et al\textsuperscript{145} using a similar assay, the only finding that correlated with outcome was the sensitivity of the clonogenic cells to 4-hydro-peroxy-cyclophosphamide used to purge the autografts. More recently, Uckun et al\textsuperscript{146} reported a series of 83 patients with ALL who underwent autologous BMT. In this series, the median value for colony-
forming cells was 0.005%. Of the 42 patients with greater values before BMT, 38 subsequently relapsed, whereas 4 died of BMT-related complications. Of the 41 patients with lower values, 20 relapsed post-BMT; 10 patients of this group remained in remission 11 to 54 (median, 40) months after BMT.

DETECTION OF MRD WITH IMMUNOLOGIC METHODS

Leukemia-associated phenotypes and methodological considerations. Single antibodies are not suitable for distinguishing neoplastic from normal lymphohematopoietic cells, because the same antigens found on malignant cells are also present on their normal counterparts. Possible exceptions to this rule are the chimeric proteins that result from chromosomal translocations and gene fusions, such as BCR-ABL, E2A-PBX1, and ALL-1-AF4 (Table 1). Antibodies reacting specifically with these tumor-associated antigens would, in principle, allow single-color flow cytometric studies of MRD, but such reagents suitable for immunofluorescence staining of patients' samples are not yet available. However, a recently developed monoclonal antibody (named 7.1) appears to specifically recognize cells from cases defined by 11q23 abnormalities. In preliminary studies, the 7.1 antibody has proved sufficiently sensitive to allow detection of 1 leukemic cell among 10,000 normal hematopoietic cells with use of flow cytometry (E. Coustan-Smith, D. Campana, F.G. Behm, unpublished results). Mori et al have recently described a reagent (KOR-SA3544) that appears to specifically recognize, among lymphoid cells, leukemic lymphoblasts bearing the Ph chromosome. Because this antibody also reacts with myeloid cells, it must be used in combination with reagents that identify lymphoid progenitors (eg, CD10, CD3, and anti-TdT).

Although some investigators have used markers such as CD10 and TdT to identify leukemic lymphoblasts in PB or BM, the results have generally been disappointing. This is not surprising because these markers are also expressed by normal B-cell precursors found in BM and, albeit rarely, in blood. The current strategy of detecting residual disease by immunologic methods takes advantage of the observation that leukocyte markers may be found on malignant cells in combinations that normally are not observed in PB and BM. Such phenotypes can be identified by double- or triple-color staining techniques performed with antibodies conjugated to different fluorochromes. Until recently, the expression of intracellular antigens (eg, nuclear TdT, cytoplasmic CD3) was optimally investigated by staining cyt centrifuge preparations and applying fluorescence microscopy. With the availability of novel techniques and reagents for cell fixation and permeabilization, it is now possible to detect intracellular antigens in cell suspensions and to analyze their expression by flow cytometry (Fig 3). The sensitivity of fluorescence microscopy depends on the number of cyt centrifuge preparations screened. A screening of multiple slides per sample may be necessary to achieve a sensitivity on the order of 1 leukemic cell among 10,000 normal hematopoietic cells, as shown by experiments with artificial mixtures of leukemic blasts and normal BM cells.

Flow cytometry allows a maximum sensitivity of detection of 1 target cell in 10⁴ after repeated and prolonged cleansing of the fluids system. A more realistic sensitivity for practical applications is 1 target cell in 10⁵. Both morphologic and phenotypic features of cell populations selectively captured from a large number of BM cells can be studied by multiparameter flow cytometric analysis. By relying on the cells' light scattering properties, in addition to their immunophenotype, one can identify different morphologic subsets of BM cells with a precision not inferior to that of an expert hematopathologist observing a Romanovsky-stained smear.

Some immunophenotypic combinations present on leukemic cells are confined to certain normal tissues and are not found in BM, PB, or cerebrospinal fluid (CSF). For example, in most cases of T-ALL, the lymphoblasts express nuclear TdT in association with T-cell markers such as CD3, CD5, or CD7. Although normally expressed by developing T cells, such phenotypes are usually not found outside the thymus. Thus, detection of individual TdT⁺ cells expressing these T-cell–associated markers in the blood or BM of T-ALL patients indicates residual disease. Another useful illustration is provided by TdT-positivity in the spinal fluid. Because this marker is not present on cells that normally migrate to the central nervous system (CNS), its presence in spinal fluid can be taken as evidence for leukemic meningeal infiltration.

In a fraction of B-lineage ALL and AML cases, the leukemic cells express combinations of normal differentiation antigens that are extremely rare or absent among normal
hemopoietic cells.\textsuperscript{156,173-178} Hurwitz et al\textsuperscript{177} reported "asynchronous" combinations of cell differentiation antigens in 78\% of cases of B-lineage ALL. In our experience, only a few of these markers are useful for truly sensitive detection of MRD (ie, at the 1:10\(^4\) level; see Table 4). Measurements of the intensity of antigen expression by flow cytometry can also be helpful.\textsuperscript{116,117} In 75\% of their large series of AML cases, Adriaansen et al\textsuperscript{127} detected subsets of cells that expressed myeloid markers and TdT. However, in 32 of these 45 cases, the TdT\(^+\) cells represented less than 20\% of the cell population. More recently, Reading et al\textsuperscript{116} reported unusual coexpression of antigens in 85\% of 272 cases of AML studied with a panel of 22 antibodies. Among the various phenotypic combinations suitable for study of residual disease in AML, the phenotype CD34/CD56 has emerged as one of the most informative.\textsuperscript{110} It is found in approximately 20\% of cases of childhood AML and is especially frequent in cases with the t(8;21)(q22;q22).\textsuperscript{119} In contrast, among normal CD34\(^+\) cells, CD56 is either undetectable or expressed at much lower levels than in leukemic blasts.\textsuperscript{118,119}

Table 4 summarizes the phenotypic combinations that can be used for productive study of residual ALL. The vast majority of T-ALLs are amenable to immunophenotypic analysis. Some B-lineage ALL cases express more than one phenotype suitable for analysis of residual disease; therefore, the overall proportion of childhood cases that can be investigated with these combinations is approximately 35\%. In most cases, intensity of expression may also help in distinguishing leukemic cells from the rare normal progenitors expressing these phenotypes. False-positive results with immunologic methods can derive from expression of leukemia-associated phenotypes by small subsets of normal cells and from the use of antibodies with nonspecific reactivities, such as cell binding through their Fc portion, a common feature of murine antibodies of IgG2 isotype.\textsuperscript{115} The former problem can be resolved with the use of multiple combinations of reagents identifying different leukemia-associated phenotypes in each case; the latter can be prevented by careful selection of optimal reagents and by saturation of Fc receptors with human or rabbit Ig. Phenotypic switches that occur at the time of relapse may result in false negative during monitoring of MRD if these affect the markers included in the leukemia-associated phenotypes studied.\textsuperscript{114,118} The investigation of multiple phenotypes in each case may help reduce the problem.

**Clinical studies.** Bradstock et al\textsuperscript{161} were the first to report the use of leukemia-associated phenotypes to investigate MRD. Among 18 patients with T-ALL, they identified 6 whose remission BM samples contained 0.5\% to 5\% nucleated cells with an abnormal phenotype identical to that observed at diagnosis (T-cell marker in conjunction with nuclear TdT and the lack of major histocompatibility complex [MHC]-class II antigens). This observation provided one of the first indications that leukemic cells could be detected by immunologic methods in remission BM samples. The reliability of this strategy was confirmed by Van Dongen et al,\textsuperscript{120} who used double-color combinations including a T-cell marker (CD5) and TdT to detect MRD in BM or PB samples of 26 T-ALL patients. Of the 12 relapses that developed in 10 patients, 11 were detected immunologically 4 to 21 weeks earlier than by routine morphologic examination. None of the remaining 16 patients without detectable CD5/TdT double-labeled cells relapsed during a median follow-up of 43 months. Drach et al\textsuperscript{166} studied four patients with T-ALL, none of whom had detectable leukemic cells by flow cytometry and all of whom remained in continuous complete remission with a median follow-up of 30 weeks after the last negative assay. Suspicious cells were observed in two of four evaluable cases of B-lineage ALL. One of these patients subsequently relapsed, whereas, in the other, phenotypically abnormal cells progressively dissipated with continuation of remission until death from infection. The remaining two patients enjoyed continuous complete remissions accompanied by lack of immunologically detectable leukemic cells (follow-up time, 20 and 24 weeks after the negative finding). In one of our earlier studies, MRD detection preceded overt relapse by 1 to 6 months in all 15 ALL patients; of the 22 patients considered to be in clinical and immunologic remission, only 7 subsequently relapsed, 2 in the CNS.\textsuperscript{168} Similar results are being obtained in an ongoing study at St. Jude Children’s Research Hospital in which 7 of 10 patients with positive immunologic assay results during continuation

<table>
<thead>
<tr>
<th>Disease</th>
<th>Phenotype</th>
<th>Frequency (%)</th>
<th>Frequency in Normal BM</th>
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<tr>
<td>B-lineage ALL*</td>
<td>TdT-CD10 (or CD19-CD34)/CD13</td>
<td>7</td>
<td>0.02 ± 0.01</td>
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<tr>
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<td>TdT-CD10 (or CD19-CD34)/CDw65</td>
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<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>TdT-CD10 (or CD19-CD34)/CD21</td>
<td>10</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>TdT-CD10 (or CD19-CD34)/CD56</td>
<td>9</td>
<td>&lt;0.01</td>
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<td></td>
<td>TdT/cytoplasmic μ/CD34</td>
<td>14</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>T-ALL</td>
<td>TdT/cytoplasmic CD3</td>
<td>90</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AML</td>
<td>CD34/CD56</td>
<td>20</td>
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</tr>
<tr>
<td></td>
<td>CDw65/CD34/TdT</td>
<td>15</td>
<td>&lt;0.01</td>
</tr>
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* Approximately 35\% of cases have at least one leukemia-associated phenotype.
† Greater than 10\% positive leukemic lymphoblasts.
‡ ALL- and AML-phenotypes were studied using light-scattering gates typical of immature lymphoid and myeloid cells, respectively. The reduced level of expression of these phenotypes by normal cells makes it possible to distinguish 1 leukemic cell among 10,000 normal BM cells by relying on such antigens in MRD assays.
therapy relapsed 1 to 7 months later, as compared with 10 relapses among 90 patients with a negative test (Campana and D. Campana et al, unpublished results).

Studies of MRD by immunologic methods have also extended to AML. Adriaansen et al studied 12 TdT+ AML patients during morphologic remission, 7 of whom subsequently developed one or two relapses (total of 10 relapses). Nine relapses were preceded by a gradual increase in phenotypically abnormal cells. The false-negative results in 1 case were probably caused by a phenotypic shift, because cells were TdT-negative at the time of overt relapse. The remaining 5 patients stayed in complete remission, with a follow-up of 32 to 46 months. Although small proportions of phenotypically abnormal cells (<0.1% in the BM) could be detected in these patients, they did not increase with serial sampling. Of the five AML patients in morphologic remission who were studied by Drach et al, three showed the persistence or increase of phenotypically abnormal cells, followed by overt relapse. In our earlier study, immunologic evidence of MRD was found in 4 of 7 patients with AML, all of whom subsequently relapsed. More recently, we have studied 13 children in complete remission after BMT (Campana and D. Campana et al, unpublished results). The 4 patients with detectable leukemic cells by flow cytometry relapsed within 2 months after the positive test. By contrast, 7 of 9 patients without detectable disease in repeated studies remained in remission, with a median follow-up of over 1 year after transplantation. Two patients relapsed despite having negative immunologic test results. As in the study by Adriaansen et al, the false-negative results were probably caused by phenotypic switches, because the markers used were not expressed at the time of relapse.

DETECTION OF RESIDUAL DISEASE IN CSF

Approximately 5% to 10% of patients with ALL who enter remission will have an isolated CNS relapse. Some of these patients may also have residual leukemia in the BM, as shown by recent studies in which most children with ALL and "isolated" CNS relapse were found to have molecular evidence of MRD in the BM. The first sign of CNS involvement is often the presence of malignant cells in the CSF, although it may be difficult to discriminate between leukemic lymphoblasts and normal activated lymphocytes by routine morphologic examination. Immunologic methods can resolve this ambiguity. Although B- and T-lymphocytes and monocytes are normally found in the CSF, cells expressing nuclear TdT should be absent. Thus, detection of even a single TdT+ cell would indicate CNS leukemia. Hooijkaas et al reported the results of a 5-year follow-up study in which anti-TdT staining was applied to the analysis of CSF from 113 children with TdT+ acute leukemia or T-cell non-Hodgkin’s lymphoma. In approximately 8% of the samples, there was discordance between the results of morphologic and immunologic evaluation. In discordant cases, the results of immunologic investigations appeared to be more informative than morphologic examination in predicting clinical outcome. In our own study of over 1,200 samples, the frequency of disagreement between morphology and immunology was lower, 1.9% (E. Coustan-Smith, D. Campana et al, unpublished results). Thus, although routine examination of CSF samples by immunologic methods would probably not be cost-effective, this approach can lend a diagnostic edge when morphologic findings are equivocal.

SUMMARY AND PERSPECTIVE

A recurring challenge in the treatment of childhood ALL is how to identify patients who will need additional and more aggressive therapy to avert relapse. Prognostic factors such as the initial leukocyte count, lymphoblast genetic features, and age of the patient at diagnosis are useful in this regard, but they do not correspond to the course of leukemia as closely as one would like. Thus, the ability to detect residual leukemic cells while they are still sensitive to treatment would allow more timely, and potentially curative, intervention. The persistence of small numbers of leukemic cells well into the clinical course is a defining feature of childhood ALL, requiring prolonged therapy (2.5 to 3 years) to reduce the relapse hazard. Whether early detection and eradication of MRD would lead to higher cure rates is a key issue that merits controlled clinical evaluation.

The therapeutic picture in childhood AML and in adult patients with either lymphoid or myeloid acute leukemia is much different. Only approximately a third of these patients can be expected to survive beyond 3 years on available multiagent chemotherapy, and the results of retrieval treatment are dismal. One of the best options for these patients is probably allogeneic or autologous BMT. The latter procedure offers the greatest potential for advancing cure rates because it does not require an HLA-matched donor. However, its utility continues to be limited by a high rate of disease recurrence, which is contributed to, at least in part, by residual leukemic cells in the BM implant. Studies of MRD could provide means of detecting such cells in "remission" BM samples and in PB autografts.

Investigators can choose from many different options when attempting to monitor patients for MRD (Table 5). The T-ALL subgroup is particularly suitable for MRD studies because several sensitive and reliable methods can be applied to study the majority of cases with this phenotype. Each of the available techniques has its own merits and limitations, which should be carefully weighed before a selection is made. For example, in many (but not all) studies by PCR, residual lymphoblasts are readily detectable immediately after remission induction and, in a substantial proportion of patients, up to 24 months postremission; however, their presence does not necessarily correlate with long-term responses to treatment (Table 3). By contrast, immunologic techniques for detecting MRD appear to identify more clearly patients at a higher risk of relapse. A simple explanation for this discrepancy is that there are differences in sensitivity between the two methods, although it could be argued that the detection level routinely passed by PCR, one would also expect a higher proportion of false-negative results by immunologic assays, but this has not been the case in the studies performed to date.
DETECTION OF MINIMAL RESIDUAL DISEASE

Table 5. Methodologic Options for Detecting MRD in Patients With Acute Leukemia

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Method</th>
<th>Potentially Suitable Cases (%)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL B-lineage</td>
<td>In situ hybridization</td>
<td>25-35</td>
<td>?</td>
</tr>
<tr>
<td>B-lineage</td>
<td>Ploidy determination</td>
<td>5-25</td>
<td>?</td>
</tr>
<tr>
<td>T-ALL</td>
<td>PCR on translocation breakpoints</td>
<td>10-30</td>
<td>10^-4-10^-6</td>
</tr>
<tr>
<td>ALL</td>
<td>Southern blotting for Ig or TCR genes</td>
<td>&gt;90</td>
<td>10^-2</td>
</tr>
<tr>
<td>ALL</td>
<td>PCR on TCRy and TCR6 genes</td>
<td>40-60</td>
<td>10^-3-10^-6</td>
</tr>
<tr>
<td>ALL</td>
<td>PCR on IgH genes</td>
<td>&gt;90</td>
<td>10^-3-10^-5</td>
</tr>
<tr>
<td>ALL</td>
<td>Colony assays</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>ALL</td>
<td>Immunophenotyping</td>
<td>35</td>
<td>10^-4</td>
</tr>
<tr>
<td>T-ALL</td>
<td>In situ hybridization</td>
<td>10-20</td>
<td>?</td>
</tr>
<tr>
<td>T-ALL</td>
<td>Ploidy determination</td>
<td>&lt;5</td>
<td>?</td>
</tr>
<tr>
<td>AML</td>
<td>PCR on translocation breakpoints and TAL-1 deletions</td>
<td>25-35</td>
<td>10^-4-10^-5</td>
</tr>
<tr>
<td>AML</td>
<td>Southern blotting for TCR genes</td>
<td>90-95</td>
<td>10^-2</td>
</tr>
<tr>
<td>AML</td>
<td>PCR on TCRy and TCR6 genes</td>
<td>40-60</td>
<td>10^-3-10^-5</td>
</tr>
<tr>
<td>AML</td>
<td>Colony assays</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>AML</td>
<td>Immunophenotyping</td>
<td>90-95</td>
<td>10^-4</td>
</tr>
</tbody>
</table>

Abbreviation: ?, not known.

Table 6. Potential Sources of Error in Detection of MRD

<table>
<thead>
<tr>
<th>Method</th>
<th>False-Negative Result</th>
<th>False-Positive Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>Limited number of cells available for study; heterogeneous distribution of leukemia</td>
<td>Natural occurrence of aneuploidy in normal cells; artificial colocalization of probes</td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>Poor hybridization signals</td>
<td>Contamination</td>
</tr>
<tr>
<td>PCR performed on translocation breakpoints</td>
<td>Degraded RNA or DNA; lack of mRNA expression in clonogenic cells; low efficiency of RT</td>
<td>Cross-hybridization with similar sequences derived from normal lymphoid cells</td>
</tr>
<tr>
<td>PCR performed on antigen-receptor gene rearrangements</td>
<td>Degraded DNA; clonal change in rearrangement; oligoclonality</td>
<td>Colony formation from nonleukemic cells</td>
</tr>
<tr>
<td>Colony assays</td>
<td>Low proliferative activity of leukemic cells; apoptosis due to lack of stromal support</td>
<td>Antibody cross-reactivity; normal cells expressing leukemia-associated phenotypes</td>
</tr>
<tr>
<td>Immunologic methods</td>
<td>Phenotypic switch</td>
<td></td>
</tr>
</tbody>
</table>

sponsible for the observed discrepancies (an issue to be confirmed in prospective studies), accurate quantitative analysis by PCR will be critical to improve the clinical usefulness of this approach.

In addition to potential sources of error specific to each technique (Table 6), the sensitivity of all MRD detection methods is limited by the number of cells that can be analyzed and, possibly, by uneven dissemination of the disease. A BM sample taken from a typical patient with acute leukemia in "remission" yields 5 x 10^7 or fewer mononuclear cells, and, because of technical constraints, the number of cells studied is often less than 5 x 10^5. The heterogeneous distribution of leukemic cells throughout the body is familiar to oncologists, having been observed in both humans and animal models. Consequently, some residual disease can escape detection even with exceptionally sensitive techniques.

Current clinical studies of MRD should establish its clinical significance, ie, whether it predicts relapse uniformly and, therefore, justifies intensification of therapy in positive cases, or whether it simply detect leukemia cell populations whose proliferative potential has been altered by chemotherapy. Failure to relate MRD to prognosis in these studies could be interpreted in either of two ways. It could signify that some patients have low levels of leukemic blasts that do not invariably cause relapse, which would indicate, further, that one may not need to eradicate the entire leukemic cell population to effect cure. Alternatively, it might be argued that sampling intervals, imposed by practical constraints, are not frequent enough to permit reliable detection of MRD; therefore, it is important that each critical point in a patient's disease course is covered by BM sampling. For example, in ALL we suggest that samples should be taken at least at the following points in all studies: (1) the end of remission induction, when cells with intrinsic drug resistance are beginning to emerge; (2) the end of the first year of therapy, after which most relapses during treatment occur; and (3) immediately before cessation of therapy, when the more indolent leukemic blasts responsible for delayed relapse are likely to be found.

In conclusion, accurate determination of MRD may have profound impact in the clinical management practices of patients with hematologic malignancies. The technologic developments and the preliminary clinical results warrant prospective studies of large cohorts of homogeneously treated patients using a multidisciplinary approach. The definition of the clinical importance of MRD at different stages of treatment is the first goal to be pursued by these studies, the success of which depends on the close collaboration between laboratory investigators and clinicians.
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