The Immunologic Detection of Minimal Residual Disease in Acute Leukemia

By Dario Campana, Elaine Coustan-Smith, and George Janossy

RESIDUAL DISEASE in patients with acute leukemia indicates unfavorable prognosis. Nevertheless, it is difficult to identify less than 5% malignant cells in cases other than L3 leukemias by morphology alone, and a tumor burden of 10³ cells might remain unnoticed.1 Many techniques such as cytogentic,2 the analysis of gene rearrangements,3,4 colony assays,5 and immunologic analysis of differentiation antigens are suitable to complement the morphologic definition of remission. Of these methods, one of the most precise is the use of double-color immunofluorescence (IF), introduced in 1979 for detecting residual blasts in the bone marrow (BM) of thymic acute lymphoblastic leukemia (T-ALL) patients.6,7 In normal tissues, cells expressing both T-cell associated antigens and nuclear terminal deoxynucleotidyl transferase (TdT) are limited to the thymus6 and are absent from normal peripheral blood (PB) and BM.6,8 A similar concept has also been applied for detecting ALL blasts in the cerebrospinal fluid where a single TdT+ cell may indicate leukemic infiltration of the central nervous system.6,10

During the following years the leukemic blasts’ phenotype has been characterized by monoclonal antibodies (MoAbs), and the normal differentiation pathways of T and B lymphoid as well as myeloid lineage cells have been defined.11,12 On the one hand, these studies have confirmed that leukemic cells reflect features of lymphohematopoietic precursor cells13-16 such as the presence of TdT, CD19, and cytoplasmic CD22 in B-lineage ALL and of TdT and cytoplasmic CD3 (cCD3) in T-lineage ALL. On the other hand, the expression of membrane, cytoplasmic, or nuclear antigens in a proportion of leukemias shows signs of asynchrony.17,18 Thus, double-staining combinations may show diagnostically useful deviations from normality. The possibility of applying these assays for detecting residual disease is also indicated by their sensitivity: mixing experiments performed in our laboratory19,20 and by others21,12 have shown that 10⁻⁴ doubly labeled cells can be reliably detected on cytocentrifuge preparations using fluorescence microscopes equipped with selective filters for different fluorochromes and phase contrast attachments.

Although the technology is well-established, concern has been raised about its clinically informative value. Problems due to false-positive observations and lack of sensitivity could emerge in a clinical setting, and variations in leukemia distribution could also render these assays insensitive. To address these issues, we have first defined leukemia-associated phenotypes by comparing the phenotypic features of leukemic blasts to those of normal progenitor cells. Some of these, such as the expression of T-cell associated markers and TdT in T-ALL blasts have been known (see above), but the frequency of other unusual combinations such as the coexpression of CD13 or CD33 myeloid antigens on normal TdT+ cells in fetal and regenerating samples have not yet been fully investigated. Second, we compared the phenotypes of leukemia at presentation and in relapse to assess the incidence of phenotypic changes influencing the reliability of double IF assays. Third, we compared morphologic and immunologic techniques in 250 posttreatment BM samples. Finally, we tested the specificity of these assays by investigating retrospectively the outcome of a group of acute leukemia patients in complete morphologic remission. In 19 of these cases, second, the outcome of 19 patients with minimal disease identified immunologically while in complete morphologic remission was investigated: all 19 patients have developed systemic relapse within 4 to 25 (median 14.5) weeks. In contrast, 17 of 25 patients also morphologically in complete remission and without residual disease identifiable immunologically after repeated testing are still in morphologic and immunologic remission (follow-up 17 to 114 weeks, median 28 weeks). Only eight patients in this group have relapsed so far: in two patients the relapse was localized in the cerebrospinal fluid, while in six patients a systemic relapse was observed 6 to 51 (median 21.5) weeks after the last negative immunologic bone marrow examination. In conclusion, no false-positive results were detected with these sensitive assays, and the introduction of appropriately planned prospective studies, including the immunologic detection of residual leukemia, is justified on the basis of these observations.

© 1990 by The American Society of Hematology.
patients, cells exhibiting the leukemia-associated phenotype established at presentation were identified in the remission BM, while in 25 other patients such cells were not seen. We demonstrate that the presence of cells with leukemia-associated phenotypes is invariably followed by full morphologic relapse.

MATERIALS AND METHODS

Handling of samples. Human fetal tissues were obtained after legal termination of pregnancy. Gestational age was calculated from foot and, when possible, crown/rump length and range from 13 to 20 weeks. Fetal liver tissue was teased in cold RPMI-1640 medium and washed three times in phosphate-buffered saline (PBS). Regenerating BM samples were from patients with acute leukemia in complete remission 1 to 6 months after cessation of chemotherapy or following BM transplantation. Infant BM samples were from 3- to 20-month-old patients with no evidence of malignancy.

PB and BM samples of patients with ALL and acute myeloid leukemia (AML) were taken with informed consent and sent to our laboratory for immunodiagnosis and follow-up after remission induction or BM transplantation.

Mononuclear cells were separated on Ficoll-Hypaque density gradient and washed three times in PBS. The viability, assessed by trypan blue dye exclusion, was greater than 90% in the adult and infant samples, and greater than 80% in the fetal samples included in this study.

Staining of cells. The techniques were performed according to the recommendation of the International Committee for Standardization in Hematology. The MoAbs used in this study are listed in Table 1. Double-color IF was performed by using second-layer antisera to immunoglobulin (lg) of different species. These were conjugated to different fluorochromes such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC).

Table 1. MoAbs and Heterologous Antisera Used in This Study

<table>
<thead>
<tr>
<th>Name</th>
<th>CD</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoAbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFAL 1</td>
<td>10</td>
<td>RFH&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>RFB9</td>
<td>19</td>
<td>RFH</td>
</tr>
<tr>
<td>RFB4</td>
<td>22</td>
<td>RFH</td>
</tr>
<tr>
<td>RFB7</td>
<td>37&lt;sup&gt;+&lt;/sup&gt;</td>
<td>RFH</td>
</tr>
<tr>
<td>RFT11</td>
<td>2</td>
<td>RFH</td>
</tr>
<tr>
<td>UCHT1</td>
<td>3</td>
<td>Professor P.C.J. Beverley, Middlesex Hospital, London, UK</td>
</tr>
<tr>
<td>RFT2</td>
<td>7</td>
<td>RFH</td>
</tr>
<tr>
<td>WMS64</td>
<td>13</td>
<td>Dr K. Bradstock, Westmead Hospital, Sydney, Australia</td>
</tr>
<tr>
<td>WM15</td>
<td>33</td>
<td>Dr K. Bradstock</td>
</tr>
<tr>
<td>Heterologous antisera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit-anti-TdT</td>
<td></td>
<td>Supertechs Inc, Bethesda, MD; Cat. No. 004</td>
</tr>
<tr>
<td>Goat-anti-mouse Ig FITC/TRITC</td>
<td></td>
<td>RFH</td>
</tr>
<tr>
<td>Goat-anti-human IgM FITC/TRITC</td>
<td></td>
<td>Southern Biotechnology Associates, Birmingham, AL; Cat. No. 2020-02/03</td>
</tr>
<tr>
<td>Goat-anti-rabbit Ig TRITC</td>
<td></td>
<td>SBA, Cat. No. 4010-03</td>
</tr>
</tbody>
</table>

<sup>*</sup>Department of Immunology, Royal Free Hospital School of Medicine, London, UK.

<sup>+</sup>RFB7 is a CD37 MoAb that reacts exclusively with B cells.

Mononuclear cells were labeled with antibodies using a microplate method previously described. Cells at a final concentration of approximately $2 \times 10^6$/mL were resuspended in PBS containing 0.2% bovine serum albumin and 0.2% sodium azide (PBSA); 50 mL of these suspensions was distributed into U-bottomed 96-well microtiter plates, and MoAbs were added using multichannel pipettes at the optimal concentration as demonstrated in preliminary titration experiments (these were 1:5 to 1:25 for culture supernatants and 1:10<sup>2</sup> to 1:10<sup>3</sup> for ascitic fluids). Careful consideration was paid to negative controls: normal mouse serum, containing a mixture of murine Ig of different isotype, was regularly used instead of MoAbs in parallel wells. After a 10-minute incubation with MoAbs or mouse serum at 20°C, cells were washed four times in PBSA and incubated with goat-anti-mouse Ig FITC for a further 10 minutes. After four washes in PBSA, a multichannel pipette was used to transfer small droplets of cell suspensions onto 12 separate areas on polytetrafluoroethylene coated slides (Henley, Essex, UK). Cells were fixed for 10 minutes in formalin vapors, air-dried, and covered with glycerol/PBS 1:1 and a coverslip. Slides were observed with a Zeiss epifluorescence microscope (Oberkochen, FRG) with selective filters for FITC and TRITC.

From the cells stained in the wells, cytofluorographic preparations were also made using a Shandon Cytopsin II centrifuge (Shandon Southern, Astmoor, Runcorn, UK). To visualize TdT, cytoplasmic $\mu$, CD3, and CD22 cytopsins were fixed in cold methanol for 30 minutes, or cold acetone:methanol 1:1 for 15 minutes, or acetone for 5 to 10 minutes at 20°C. After incubating the preparations with antibodies and second-layer antisera (30 minutes at 20°C), the slides were washed in PBS and mounted with glycerol/PBS 1:1 and a coverslip. The corresponding control slides were incubated with normal rabbit IgG (200 ng/mL) instead of rabbit-anti-TdT antibody; goat-anti-human IgM FITC or TRITC was replaced by goat antiserum to human lambda light chain conjugated to FITC or TRITC (Southern Biotechnology Associates, Birmingham, AL, Cat. Nos. 2070-02 and 2070-03), and mouse serum was used instead of CD3 and CD22 MoAbs. The concentration of IgG in the control reagent was similar to that of the corresponding MoAb.

Finally, the remaining cells in the wells were fixed with 2% to 4% formaldehyde or 0.5% paraformaldehyde, and analyzed with a FACScan (Becton Dickinson, Mountain View, CA). When nonspecific staining (eg, positivity with mouse serum) was observed, unlabeled cells were reincubated with the same reagents after 10 minutes' preincubation with rabbit serum to inhibit Fc receptor binding.

Statistical analysis. The statistical significance of the differences observed in the phenotypic features of childhood and adult ALL as well as in the outcome of patients with and without residual disease was evaluated with a chi-squared test.

RESULTS

Leukemia-associated phenotypes. It has been previously shown that the leukemic blasts of the B lineage consistently express nuclear TdT, membrane CD19, and cytoplasmic CD22 (TdT<sup>+</sup>,CD19<sup>+</sup>,cCD22<sup>+</sup>). In more than 90% of cases, greater than 60% of blasts carry these markers. In this study we have documented other unusual features on some of the leukemic cases. In 17 of 178 (9.5%) cases, more than 80% of TdT<sup>+</sup> blasts were also strongly RFB7<sup>+</sup>, a B-cell restricted MoAb of the CD37 cluster, while in 5 of 178 (2.8%) blasts were CD22 (RFT11<sup>+</sup>)<sup>+</sup>. The features of pre-B ALL were found in greater than 80% of $\mu$<sup>+</sup>, TdT<sup>+</sup> blasts in 27 of 178 (15.2%) cases, and in two B-ALL cases more than 80% of blasts were $\kappa$<sup>+</sup> and TdT<sup>+</sup>. Myeloid associated antigens detected by
CD13 and CD33 MoAbs were also tested on the same cases of adult B-lineage ALL: 19 of 178 (10.7%) and 17 of 178 (9.5%) cases were CD13+ and CD33+ on greater than 80% of blasts, respectively. Because seven of the positive cases expressed the two antigens simultaneously, the cumulative proportion of adult B-lineage ALL with greater than 80% of CD13+/CD33+ blasts was 29 of 178 (16.3%). Some of the cases studied expressed more than one unusual feature on blast cells associated to TdT positivity. These were as follows: 8 of 178 (4.5%) were cμ− and CD13+/CD33−; 4 of 178 (2.2%) were cμ−/RFB7−/+; 2 of 178 (1.1%) were sIg− and RFB7−; 3 of 178 (1.7%) were RFB7++ and CD13+/CD33−. Thus, the cumulative proportion of adult B-lineage ALL with leukemia-associated features was 63 of 178 (35.4%; Table 2).

The reactivity of these antibody combinations was also studied on childhood B-lineage ALL blasts (less than 16 years of age). From 85 samples of CD19+, TdT+, cCD22− ALL, seven (8.2%) showed strong RFB7 positivity and one (1.2%) was CD2 (RFT11)+ on greater than 80% of blasts. Within this group of leukemias 12 cases (14.1%) were pre-B-ALL with greater than 80% cμ+/TdT+ blasts, while 4 of 85 (4.7%) and 5 of 85 cases (5.9%) had blasts with myeloid-associated markers CD13− and CD33+, respectively. Because three of these cases expressed both markers, the cumulative proportion of childhood B-lineage ALL with myeloid-associated antigens is 6 of 85 (7.0%), including one case of cμ− pre-B-ALL. These results indicate that myeloid-associated antigens are less frequently found in childhood ALL than in adults (P < .05). Taken together, 25 of 85 (29.4%) of B-lineage ALL express leukemia-associated features (Table 2).

In 173 samples of AML studied (all from adults), the myeloid-associated antigens were detected by the MoAbs CD13 and CD33 on the majority of blast cells. More than 80% of blasts also exhibited CD7 antigen in 17 of 173 cases (9.8%; Fig 1) and nuclear TdT in 33 of 173 (19.1%) cases. In 8 of 173 (4.6%) cases the myeloid-associated antigens and CD7 plus TdT were expressed simultaneously; thus, the cumulative proportion of CD7 and TdT expression in AML is 42 of 173 (24.3%; Table 2). It is important to note that in these leukemias the ALL-like features such as cCD3 and cCD22 expression have been thoroughly excluded, and cytochemistry also supported the diagnosis of AML.

### Leukemia-associated phenotypes in normal tissues

The presence of leukemia-associated phenotypes was investigated in fetal liver from the 13th to the 16th weeks of gestation (11 samples); fetal BM from the 17th to the 20th week of gestation (5 samples); infant BM (6 samples); and regenerating BM (12 samples). Three to six cytocentrifuge preparations, each containing greater than 5 x 10⁶ mononuclear cells were analyzed for each sample and more than 10⁵ mononuclear cells were screened.

The proportion of TdT+ cells strongly RFB7+ was 2.5% to 22% in all the samples analyzed (Table 3). The RFB7 expression on this subpopulation of normal TdT+ cells was dim when compared with the bright labeling of sIgM+ B cells seen in the same samples. The labeling intensity of RFB7 on leukemia blasts in the positive cases was intermediate between these two normal patterns of staining. Cells expressing cμ and TdT were also present in all fetal and infant BM samples except in 1 of 5 regenerating BM samples studied, but the proportions of TdT+ cells with cμ expression were uniformly low, ranging from 0.5% to 5% of TdT+ cells (Table 3).

Table 2. Proportion of Acute Leukemia Cases Expressing Leukemia-Associated Phenotypes

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>No. of Cases Studied</th>
<th>No. of Cases With Leukemia-Associated Phenotypes*</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-lineage ALL</td>
<td>263</td>
<td>88†</td>
<td>33.5</td>
</tr>
<tr>
<td>(Children)</td>
<td>85</td>
<td>25</td>
<td>29.4</td>
</tr>
<tr>
<td>(Adults)</td>
<td>178</td>
<td>63</td>
<td>35.4</td>
</tr>
<tr>
<td>T-ALL</td>
<td>63</td>
<td>60‡</td>
<td>95.2</td>
</tr>
<tr>
<td>AML</td>
<td>173</td>
<td>42§</td>
<td>24.3</td>
</tr>
<tr>
<td>Total</td>
<td>499</td>
<td>190</td>
<td>38.1</td>
</tr>
</tbody>
</table>

*Only cases with greater than 80% of blasts expressing the leukemia-associated combination were included.
†These include TdT+ cases expressing CD13, CD33, cμ, CD37, and/or CD2 (see text).
‡All cCD3/TdT cases (see text).
§These include CD13-CD33− cases expressing TdT and/or CD7 (see text).

No TdT− cells (less than 0.01% mononuclear cells) expressing CD2 could be observed in 5 of 5 fetal liver, 5 of 5 fetal BM, and 1 of 8 regenerating BM samples studied. Interestingly, however, in 7 of 8 regenerating and in 5 of 5 infant BM samples the proportion of TdT+ cells expressing CD2 was 3% to 15% (Table 3). In regenerating BM samples studied, but not in the fetal tissues, a similar low percentage of TdT+ cells also weakly expressed CD7 antigen (Table 3; see also reference 23).

These findings indicate that normal TdT+ cells expressing RFB7, cμ, CD2, and/or CD7 are found in low frequencies (less than 1% to 20% of TdT+ cells; Table 3); when greater than 20% TdT+ cells expressing such molecules are seen this might indicate the presence of residual leukemic blasts. In contrast, the analysis of TdT+ cells in fetal, infant, and regenerating BM showed the total absence (less than 0.01% mononuclear cells) of TdT+, CD13− and TdT+, CD33− double positive (Table 3). Thus, with such antibody combinations, 0.01% residual leukemic cells can be detected. Also absent were TdT+ cells expressing cytoplasmic or membrane CD3 (Table 3, Fig 2) as already reported.13,24 Cells with such phenotype are confined to the thymic gland, and even a single CD3+/TdT+ cell amongst more than 10⁶ normal PB or BM cells may be taken as an indication of leukemic infiltration.

### Phenotypic switches in relapse

Repeated samples were studied in 35 patients, including 10 B-lineage ALL, 16 T-ALL, and 8 AML (Table 4). One acute undifferentiated leukemia (AUL) with TdT, CD7, and class II antigen expression but no lymphoid or myeloid features was also studied: these blasts lacked cCD22, CD19, cCD3, CD13, CD33, CD61, and glycoporphin expression (Table 4). These patients were selected because the presentation samples showed leukemia-associated phenotypic features; no patient with blast crisis of chronic granulocytic leukemia was in-
The detection of residual leukemia with multiparameter analysis in flow cytometry. Light scattering (A) and double-color IF (B) analysis of one AML case identified leukemia-associated features at presentation: 84% of blasts (see gating in A) simultaneously expressed CD7 and CD33 (indicated in B as FI.1 and FI.2, respectively). No blasts expressing the same features are seen in normal BM (C), even after analyzing greater than 10^6 cells. Mixtures of AML and normal bone marrow cells were prepared, labeled with CD7 and CD33 MoAbs, and studied after setting the gates as in A and B. The analysis of samples containing 0.1% and 1% of AML blasts is illustrated in D and E, respectively: 42% and 44% of total cells fell in the blast light scattering gate; of these, 0.2% and 1.9% simultaneously expressed CD7 and CD33. In these experiments 0.01% residual AML could be detected after analyzing 5 \times 10^6 cells. The leukemia associated features of this AML case were retained in the relapse sample, received 6 months later (F).

ccluded in this study. All samples showed TdT positivity in greater than 80% of blasts at presentation.

Important phenotypic shifts were observed in two cases. Loss of TdT in relapse was detected in 1 of 34 TdT+ leukemias studied. In 1 of the 4 cases of CD10+, TdT- pre-B-ALL, the relapsing blasts failed to express CD10. None of the remaining six cases of B-lineage ALL with myeloid antigen expression changed their phenotype during relapse. Similarly, the eight cases of TdT+ and/or CD7+ AML studied remained unchanged in relapse. The blasts in 15 of 16 cases of T-ALL studied showed no changes in relapse in respect of the expression of the crucial markers including TdT, cCD3, and CD7. Finally, in AUL the TdT and CD7 positivity remained unaltered when reinvestigated in relapse. It is relevant that other types of phenotypic changes have been observed in the same group of patients: these were the loss of CD10 expression in one T-ALL, the loss of CD8, CD4, and CD6 in another T-ALL, and the acquisition of CD33 expression in a case that lacked this antigen at presentation and was therefore recorded as AUL.

Table 3. Expression of Leukemia-Associated Phenotypes on Normal B-Cell Progenitors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TdT^+</th>
<th>CD2^+</th>
<th>CD7^+</th>
<th>CD37^+</th>
<th>cu^-</th>
<th>cCD3^+</th>
<th>CD13^+</th>
<th>CD33^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal liver and BM (9-20 wks)</td>
<td>1-21^*</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>4-11</td>
<td>2-4</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>(16)†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant and regenerating BM</td>
<td>0.8-22</td>
<td>&lt;0.1-15^†</td>
<td>&lt;0.1-8^§</td>
<td>2.6-22</td>
<td>&lt;0.1-7</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>(18)</td>
<td>(11)</td>
<td>(16)</td>
<td>(9)</td>
<td>(8)</td>
<td>(15)</td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
</tbody>
</table>

*Percentage of positive mononuclear cells. The range is indicated.
†No. of samples tested.
‡In one sample no double-labeled cells were seen (less than 1 in 10^4).
§In three samples no double-labeled cells were seen (less than 1 in 10^4).
‖In one sample no double-labeled cells were seen (less than 1 in 10^4).
*Rare cells with such phenotypes, seen in a minority of adult BM samples, have been reported (see references 29 and 30).
Cells expressing leukemic phenotypes during morphologic remission. Having established that certain marker combinations are leukemia-associated and remain stably expressed in the majority of relapsing cases, we investigated the sensitivity of these methods and asked whether the particular leukemia-associated phenotypes are detectable when the patients are considered to be in complete morphologic remission by two experienced hematologists. Although such combinations are present on greater than 80% of blasts only in 35% to 40% of acute leukemia patients, it is rewarding to investigate the quality of remission and the pattern of relapse in these selected cases. In the course of the study, 250 BM samples from patients with treated leukemia were found to be in full morphologic remission with no excess of blast cells. Of these, 73 samples (including 12 with positive immunologic findings; see below) were also reexamined with May-Grünwald Giemsa staining in cytocentrifuge preparations after the enrichment of mononuclear cells, but were again judged to be apparently free of leukemia.

In 29 of 250 (11.6%) samples, cells with leukemia-associated combinations were found. Other phenotypic changes have been observed in some of these patients (see text). The changes in leukemia-associated combinations are shown.

### Table 4. Occurrence of Phenotypic Changes in Relapse

<table>
<thead>
<tr>
<th>Type of Leukemia*</th>
<th>Phenotype at Presentation</th>
<th>Change in Expression of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TdT</td>
</tr>
<tr>
<td>B-lineage ALL</td>
<td>CD13-CD33/TdT</td>
<td>0/6</td>
</tr>
<tr>
<td>Pre-B ALL</td>
<td>cu/TdT</td>
<td>0/4</td>
</tr>
<tr>
<td>T-ALL</td>
<td>CD3/TdT</td>
<td>1/16</td>
</tr>
<tr>
<td>AML</td>
<td>CD13-CD33/TdT</td>
<td>0/7</td>
</tr>
<tr>
<td>AUL</td>
<td>CD13-CD33/CD7</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1/34</td>
</tr>
</tbody>
</table>

Changes in leukemia-associated combinations are shown. Other phenotypic changes have been observed in some of these patients (see text).

*Only cases with greater than 80% of blasts showing a leukemia-associated phenotype were included.
associated features were detected. These aberrant cells represented up to 10\% of the mononuclear fraction, the lowest values being 1 and 4 cCD3/TdT+ cells (less than 0.01\%) in three cytocentrifuge preparations screened (Table 5; Fig 2). In 88.4\% of samples no leukemia was identifiable with methods that are potentially capable of detecting 104 leukemic contamination.

**Detection of early relapse.** Next we investigated the specificity of these findings and asked whether the identification of leukemia-associated phenotypes in BM samples may indicate relapse. For this retrospective analysis we selected 44 patients according to the following three criteria: (1) leukemia-associated phenotypes at presentation; (2) full morphologic remission; and (3) the patient management, based solely on morphologic evaluation. In 19 patients (12 T-ALL, 3 B-lineage ALL, and 4 AML) 10 to 49 (median 22) years old, cells expressing leukemia-associated phenotypes were detected despite the absence of morphologic signs of relapse. These aberrant cells represented 0.02\% to 5\% of the mononuclear population and were frequently of small or intermediate size (Fig 2, G through I). In this group 16 patients were in first morphologic remission at the time of immunologic analysis, while the remaining three patients were in second remission. Two of the patients in first remission were studied after allogeneic BM transplantation. Positive immunologic findings were observed 1 to 12 (median 8) weeks after completion of remission induction chemotherapy or BM transplantation. All 19 patients relapsed after 4 to 25 (median 14.5) weeks from the initial positive immunologic finding (Fig 3). As the morphologic relapse invariably followed the immunologic findings, we conclude that in our study no false-positive observations were made. No apparent correlation was observed between the proportion of cells expressing the leukemia-associated phenotypes and the rapidity of the subsequent full morphologic relapse.

In the other 25 patients (13 T-ALL, 9 B-lineage ALL, and 3 AML) 4 to 54 (median 21) years old, no cells with leukemia-associated features were detected during the repeated testing of BM despite investigating more than 106 cells on each occasion with the relevant reagent combination.

---

**Table 5. Detection of Minimal Residual Disease in Samples Morphologically in Complete Remission**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Combination</th>
<th>No. of Samples*</th>
<th>Residual Leukemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-ALL</td>
<td>cCD3/TdT†</td>
<td>21/165</td>
<td>&lt;0.01-10</td>
</tr>
<tr>
<td>B-lineage ALL</td>
<td>CD13/TdT</td>
<td>4/31</td>
<td>0.02-5</td>
</tr>
<tr>
<td>Pre-B-ALL</td>
<td>cu/TdT</td>
<td>0/14</td>
<td>—</td>
</tr>
<tr>
<td>AML</td>
<td>CD13/TdT</td>
<td>4/40</td>
<td>0.1-5</td>
</tr>
<tr>
<td></td>
<td>CD33/TdT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD7/TdT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total 29/250 (11.6\%)

In 73 samples the morphologic remission was also assessed after the enrichment of mononuclear cells.

*Some of these represent repeated investigations of the same patient: the total number of patients studied is 119.

†Double-labeled cells were often of small or intermediate size.

---

**Fig 3. The detection of early relapse with double-color immunofluorescence in patients in complete morphologic remission.** Patients were selected because of the identification of leukemia-associated phenotypes at presentation, the complete morphologic remission, and the clinical management exclusively based on the morphologic findings (see text). In the 19 MRD+ patients, leukemia-associated phenotypes were found during the immunologic investigation of the remission BM samples, while in the 25 MRD- patients repeated analysis did not show the presence of cells exhibiting such phenotypic combinations (see text). Hatched and dotted bars represent patients in which overt morphologic relapse occurred during the follow-up period (MRD+, AML relapse; MRD-, B-lineage ALL relapse; MRD-, T-ALL relapse). Empty bars (0) represent patients still in morphologic and immunologic remission. The number of patients included in each group is shown in parentheses. In the MRD+ group systemic morphologic relapse occurred 4 to 25 (median 14.5) weeks after the first detection of leukemia-associated phenotypes in the remission BM. In the MRD- group two patients had central nervous system (but not systemic) relapse and six patients relapsed 6 to 51 (median 21.5) weeks after the last BM analysis. The remaining 17 patients are in morphologic and immunologic remission, alive and well, with a follow-up of 17 to 114 (median 28) weeks (P < .001).

All the patients were in first morphologic remission at the time of the initial immunologic investigation, which was performed 1 to 24 (median 4) weeks after cessation of remission induction chemotherapy. Thus, the study of these patients was performed at a time in the course of the disease similar to that of the patients with immunologically detectable leukemia shown above. After a follow-up period of 17 to 114 (median 28) weeks, 17 of these 25 patients are well and still in complete morphologic and immunologic remission (Fig 3). The remaining eight patients in this group relapsed with blasts showing the same phenotype as in their presentation sample. Two patients of this group (1 pre-B-ALL and 1 T-ALL) had central nervous system relapse without systemic involvement 2 and 4 weeks after the negative BM study, while 6 patients (4 T-ALL, 1 pre-B-ALL, 1 AML) had systemic relapses after 6 to 51 (median 21.5) weeks of the last negative BM examination (Fig 3).

**DISCUSSION**

The purpose of this study was to identify leukemia-associated phenotypes and to use these for detecting minimal leukemia. The occurrence of leukemic features was demon-
The sensitivity of double-color IF techniques has been previously established by using artificial mixtures of normal and leukemic blasts, but the potential clinical value of these methods has not yet been demonstrated. In our study, these methods were applied to 250 BM samples considered to be in complete morphologic remission. After having identified leukemia-associated phenotypes identical to those seen at diagnosis in 11.6% of these samples, we also asked whether such findings would be invariably followed by morphologic relapse or whether at least some of these observations should be regarded as false-positive. These investigations are essential before prospective studies can be carefully planned to apply therapeutic strategies based on such assays.

Both the CD5/TdT and, more recently, the cCD3/TdT combinations have been shown to be thymocyte- and T-ALL–restricted. These latter combinations have been used by Van Dongen et al, in our study for the follow-up of T-ALL as well as T-lymphoblastic lymphoma. In this work we have also introduced the immunologic investigation of minimal disease in B-lineage ALL and AML by using the aberrant features seen in a proportion of these cases.

To assess the limitations of this analysis we have first studied the expression of asynchronous marker combinations in fetal and postnatal BM samples. The first of these tests includes TdT/CD13, TdT/CD33, CD7/CD13, and CD7/CD33. These are useful for both the detection of common ALL with myeloid features and that of AML with TdT and/or CD7 expression. Among the TdT+ and CD7+ populations of the normal and regenerating BM, we were unable to identify CD13+ and CD33+ cells (less than 0.01%; Table 2); thus, these tests are potentially useful for detecting early relapse. Nevertheless, in a minority of BM samples taken from patients during maintenance or off therapy or from normal adults, a few TdT+ cells expressing CD13 and/or CD33 (less than 0.05% of mononuclear cells) have been reported. From all of these studies it appears that such rare cells are preferentially found in resting samples and they are absent or extremely rare amongst actively dividing lymphohaematopoietic progenitors. As a caveat, in these tests single double-positive cells should not be considered as leukemia, and only a cohort of CD13+ /CD33+. TdT+ cells (greater than 5% of the TdT+ population) are reported as minimal disease.

Some other markers of asynchrony such as cμ/TdT and CD37/TdT are less valuable in minimal disease detection and require the assessment of the proportion of positive cells within the TdT+ population. This is because in normal samples transitional pre-B cells with double cμ/TdT expression were seen representing less than 10% of the TdT+ population, and normal TdT+ cells weakly expressing RFB7 have also been found. However, the presence of minimal disease might be considered when the cμ+ cells represent greater than 20% of the TdT+ cells seen.

The second predictable limitation of this technology, ie, the loss of antigen expression and lineage switching in relapsing leukemias, has also been investigated but was infrequent with the particular markers used in our study: only two such changes were seen among 35 patients studied. Other phenotypic features, not relevant for discriminating leukemic blasts among normal progenitors, have instead changed more frequently in patients with relapsing leukemia.

The third potential weakness of the application of immunologic methods for detecting leukemic cells is the possibility that dividing leukemic population might show a different phenotype from bulk leukemia. This is unlikely to be the case because it has previously been documented that in ALL both resting and Ki67+, bromodeoxyuridine (BrdU) incorporating proliferating blasts express identical phenotypic profiles, and results in our laboratory also indicate that in cases of AML with aberrant features, Ki67+, BrdU+ blasts also express CD13, CD33, CD7, and/or TdT (Campana D, unpublished observations).

In the past investigations where only conventional morphology was applied, the limited sensitivity of this approach has permitted speculations that after remission induction 1% to 5% malignant blasts might be or are likely to be present. This possibility is clearly excluded in the majority of cases (greater than 88%), where no residual blasts (less than 10−4) could be detected with sensitive methods. A second issue for speculation is whether the presence of residual blasts leads to imminent relapse. It appears from our study and that of the Rotterdam group that immunologically detectable minimal disease is invariably followed by the full morphologic relapse in patients on maintenance therapy or off treatment (Fig 3). These important findings, confirmed by two groups, show that during immunologic testing no apparent positivity is recorded without corresponding subsequent relapse. The immunologic tests provide an early warning, although the speed of morphologic relapse is variable: in some patients it takes as long as 2 to 5 months to develop as morphologically overt disease. The lowest numbers of residual blasts in our study were a single cCD3/TdT+ cell in one sample and four such cells in another sample during the screening of greater than 105 mononuclear cells in three cytocentrifuge preparations.

The practical limitations of the immunologic detection of residual leukemia are clear from our study. Firstly, 8 of 25 patients showed false-negative results and relapsed despite the absence of detectable BM disease. Two of these patients had a central nervous system relapse with no apparent BM involvement, and four relapsed after more than 20 weeks from the last negative BM examination. Such false-negative findings should be expected because the sample analyzed might not be representative of the leukemia distribution and may be totally devoid of malignant contamination as demonstrated by both clinical observations and animal models. Negative results may not be taken as evidence for the absence of residual disease, and the analysis of multiple samples may increase the reliability of analysis.

Secondly, only 35% to 40% of patients are suitable for such investigations, and the usefulness of other immunologic techniques for minimal residual detection remains to be explored. It has recently become feasible to identify irregularities in the intensity of antigen expression leukemia by flow
cytometry. These observations will define additional leukemia-associated phenotypic combinations exploitable for minimal residual disease detection in other patients. The detection of clonal rearrangements of IgH and T-cell receptor (TCR) genes is also potentially useful for detecting minimal residual disease in ALL, and polymerase chain reaction to amplify rearranged TCR genes has also been standardized. We envisage that the analysis of the same samples with these different methods will establish their feasibility, cost effectiveness, sensitivity, and specificity in the remission analysis. However, it is likely that the immunologic analysis described in this report will remain useful for patient monitoring due to its speed and relative simplicity. We feel that on the basis of the observations on T-ALL presented above (see also references 21 and 22), controlled trials are justified for re-treating patients in early relapse as defined by cCD3/TdT double labeling.

ACKNOWLEDGMENT

We are grateful to Dr. L. Wong, Tissue Bank, Royal Marsden Hospital, London, for providing fetal tissues, and to the clinicians entering patients into the UKALL adults and UKAML M.R.C. trials for sending samples. We also thank Dr. K.F. Bradstock, Westmead Hospital, Sydney, Australia, and Professor P.C.L. Beverley, Middlesex Hospital, London, for the gift of antibodies, and Dr. L.W. Terstappen, Becton Dickinson Monoclonal Center, Mountain View, CA, for helpful advice and discussions on the use of flow cytometry.

REFERENCES


The immunologic detection of minimal residual disease in acute leukemia [published erratum appears in Blood 1990 Nov 1;76(7):1901]

D Campana, E Coustan-Smith and G Janossy