Direct Correlation of Cytogenetic Findings With Cell Morphology Using In Situ Hybridization: An Analysis of Suspicious Cells in Bone Marrow Specimens of Two Patients Completing Therapy for Acute Lymphoblastic Leukemia

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Bone marrow cells from two pediatric patients completing therapy for acute lymphoblastic leukemia were studied using in situ hybridization with an α-satellite DNA probe specific for chromosome 17. Morphologic analysis of the end-therapy specimens from each patient had shown small numbers (7.5%, 8.5%) of cells that were suspicious for residual or recurrent disease. These cells could not be morphologically or immunophenotypically distinguished with certainty from immature lymphoid cells (hematogones), which may be present normally, sometimes in increased numbers, in the bone marrow specimens of children. In situ hybridization with a probe to chromosome 17 was used because the leukemic cells from each patient had originally been shown to have an extra copy of this chromosome. In one patient, in situ studies showed a population of cells (108 of 1,000 cells) with three hybridization signals indicating trisomy 17, and thus residual/recurrent leukemia. In the other patient trisomy 17 could not be detected. Additional hybridizations to previously stained bone marrow aspirate smears permitted a direct correlation of the cytogenetic findings with the suspicious cells on a cell-to-cell basis. The questionable cells were identified, photographed, and then re-examined after hybridization. In one patient, 13 of 18 (72%) of the suspicious cells were found to have trisomy 17, whereas in the other patient 0 of 24 (0%) demonstrated an extra copy of this chromosome. These cases illustrate a clinical application of interphase cytogenetic analysis and demonstrate how this technology can be used for direct correlation of cytogenetic findings with cell morphology. This technique should prove useful for the detection of minimal residual disease and for lineage studies in leukemia and myelodysplasia.

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THE CORRELATION of cytogenetic findings with cell morphology is not possible with conventional cytogenetic techniques. Nuclear morphology, cytoplasmic features, and membrane characteristics are all destroyed when intact cells are first arrested in metaphase and then disrupted during the preparation of metaphase cells. Teerenhovi et al. have developed techniques that permit some correlation of cytogenetic information with cytoplasmic or membrane characteristics. By using a combination of morphology, monoclonal antibodies, and chromosome studies (the MAC technique), metaphase chromosomes can be analyzed simultaneously with cytochemical or immunologic features in intact cells. Although studies with the MAC technique have provided important information regarding the nature of the cell containing the cytogenetic abnormality in a number of diseases, the technology has its limitations. Chromosome spreading and banding are far from optimal and morphologic assessment is severely limited because the nucleus is unrecognizable in metaphase.

Recently, molecular and cytogenetic techniques have made it possible to derive cytogenetic information from nonmetaphase cells, ie, from interphase and terminally differentiated cells. Through the use of chromosome-specific DNA probes or probes to specific regions of a particular chromosome, it is possible to detect numerical and even structural chromosomal abnormalities without having to analyze the chromosomes directly. Interphase cytogenetic analysis has the advantage of being rapid to use, simple to interpret, and independent of the production of high-quality metaphase spreads. Interphase cytogenetic analysis is also possible on previously stained slides. This analysis permits a direct correlation of cytogenetic findings with cell morphology as it is routinely studied microscopically.

In this report we have applied the technique of interphase cytogenetic analysis to previously stained slides to study small numbers of suspicious cells in the bone marrows of two pediatric patients who were at the end of therapy for hyperdiploid acute lymphoblastic leukemia (ALL). The ability to correlate cell morphology directly with cytogenetic findings allowed us to identify morphologically the suspicious cells in each patient and then to determine whether these cells carried a cytogenetic anomaly that had been previously demonstrated at the time of initial diagnosis. This cell-by-cell correlation of cytogenetic findings with morphology should prove useful in the detection of minimal residual disease and can provide a means for correlating cytogenetic findings with cell lineage.

CASE HISTORIES

Patient 1. Patient 1 was a 7-year-old girl who presented after a month-long illness characterized by fever, tender cervical adenopathy, progressive pallor, decreased activity, and anorexia. Physical examination was significant for asthenia, moderately enlarged tender cervical adenopathy, and a palpable spleen tip. Initial blood count showed a white blood cell (WBC) count of 19.4 × 10⁹/mm³ with 16% lymphoblasts; a hemoglobin of 7.4 g/dL, and a platelet count of 53 × 10⁹/mm³. Bone marrow aspirate yielded few nucleated cells; however, the majority were lymphoid blasts (FAB L1 morphology).

Cytochemical, immunocytochemical, and flow cytometry results...
were consistent with precursor B-cell lineage leukemia (positive for HLA-DR, CD10, CD19, and CD20; negative for myeloperoxidase, CD13, CD33, CD7, TdT, and cytoplasmic mu). Cytogenetic analysis of bone marrow showed an abnormal hyperdiploid clone: 54, XX,+X,+X,+6,+14,+17,+18,+21,+22. The patient was entered on the Children's Cancer Study Group 105 protocol for moderate-risk ALL and achieved remission. Maintenance therapy was continued for 2 years.

At the end of therapy, a routine aspiration of bone marrow was performed. The marrow aspirate was a cellular specimen that showed adequate megakaryocytes, a normal myeloid-to-erythroid ratio, and progressive maturation in all cell lines. Seven and one-half percent of the marrow cells were immature, and most of these had lymphoid features (Fig 1A). These cells ranged in size from 10 to 15 μm in diameter, although some were larger, up to 20 to 25 μm. The nuclear shapes were round or oval but, in some cells, were indented and folded, and the nuclear chromatin structure varied from coarse to dense and smudgy. Nucleoli were generally absent, although one or two indistinct nucleoli were present in some cells. These immature cells had blue cytoplasm, and occasional cells had small cytoplasmic vacuoles. These cells were regarded as suspicious for residual/recurrent disease. Cytocochenical and immunocytochemical studies showed that the cells demonstrated no myeloperoxidase or nonspecific esterase activity, and possessed the antigen CD10 (CALLA). TdT analysis was not performed. Because the suspicious cells could not be distinguished with certainty from immature lymphoid cells (hematogones) that have been reported to be present normally in the bone marrow of children, a repeat bone marrow aspirate was obtained for cytogenetic analysis.

**Patient 2.** Patient 2, a 2 and 8/12-year-old girl, presented to her pediatrician with pallor. After receiving a packed red blood cell (RBC) transfusion for a hemoglobin of 2.3 g/dL, she was transferred to a tertiary care facility. At that time, the WBC count was 5.4 × 10^9/mm^3 with 40% blasts; platelet count was 6 × 10^9/mm^3.

Physical examination was significant for pallor and enlarged liver and spleen (both palpable at the level of the umbilicus). A bone marrow aspirate and biopsy showed that marrow elements had been replaced by lymphoblasts with LI morphology. Cytocochenical, immunocytochemical, and flow cytometric analysis of the blast population showed precursor B-cell phenotype (positive for HLA-DR, CD10, CD19, CD20, and TdT; negative for cytoplasmic mu, surface Ig, myeloperoxidase, CD13, CD33, and CD7). Cytogenetic analysis of peripheral blood showed an abnormal hyperdiploid clone (55,XX,+X,+4,+6,+10,+14,+17,+18,+21,+22). Remission was induced with oral prednisone, intravenous vincristine, and doxorubicin and intramuscular L-asparaginase, and consolidation and reinfusion therapy were administered. Maintenance chemotherapy was given for 20 months after reinfusion.

A bone marrow aspirate specimen was obtained at the completion of therapy. The aspirated marrow was very cellular. The M:E ratio was normal. Granulopoiesis was unremarkable, erythropoiesis was normoblastic, and megakaryocytes were adequate. However, of concern was the finding of an increased number of immature cells with lymphoid features (Fig 1B). These cells had round to slightly irregular or indented, folded nuclei, high nuclear:cytoplasmic ratios, and modest amounts of blue cytoplasm. The nuclear chromatin was smudgy, although occasional cells had reticular chromatin with indistinct nucleoli. In general, the cells ranged in size from 10 to 15 μm in diameter, but some were larger. These cells accounted for 8.5% of the nucleated marrow elements and it could not be ascertained with certainty whether they were residual or recurrent lymphoblasts or hematogones. Small lymphocytes with more “mature” chromatin were numerous. Immunophenotyping studies were not performed, but TdT analysis showed nuclear staining in 13% of the marrow cells. A repeat bone marrow was requested for cytogenetic studies.

**MATERIALS AND METHODS**

*Specimens and controls.* The repeat bone marrow aspirates for cytogenetic studies were obtained in the usual fashion from puncture and aspiration of the posterior iliac crest. A portion of each specimen was smeared onto glass slides and the remainder was anticoagulated with heparin or EDTA and submitted for conventional cytogenetic analysis and for interphase study.

Control specimens were obtained from three women undergoing bone marrow harvest for autologous bone marrow transplantation. The primary malignancies of these patients were nonhematopoietic, and the bone marrows had been shown to be free of tumor.

*Conventional cytogenetic analysis.* Cytogenetic analysis using a trypsin-Giemsa banding technique was performed on bone marrow or peripheral blood cells obtained at the time of diagnosis, at the time of therapy, or at relapse. Metaphase cells were examined from direct preparations and from cells cultured for 24 or 48 hours without mitogens. Chromosomal abnormalities are described according to the International System for Human Cytogenetic Nomenclature.

*Interphase cytogenetic analysis.* Interphase cytogenetic analysis was performed by two methods. In one, analysis was performed on cells prepared in a manner similar to that for conventional cytogenetic study excluding the short-term cultures and Colcemid (GIBCO, Grand Island, NY) treatment. In the other method (for correlative study), analysis was performed on cells that had been previously stained with Wright’s stain.

In the first method, RBCs from the anticoagulated aspirate were lysed with ammonium chloride (0.16 mol/L). The resulting preparation was incubated with hypotonic potassium chloride (0.075 mol/L) for 8 minutes at 37°C and then centrifuged. The pellets were resuspended and then the cells were fixed (three times) with absolute methanol/glacial acetic acid (3:1 vol/vol). The fixed cells

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**Fig 1.** Suspicious cells (lymphoid-morphology blasts) in the end-therapy bone marrow aspirates in patient 1 (A) and in patient 2 (B). Morphologically, it is difficult to distinguish between hematogones and residual/recurrent lymphoblastic leukemia.
were stored at −20°C until the time of hybridization. Immediately before analysis the cells were resuspended and then dropped onto glass slides and dried in an oven at 60°C for 1 to 2 hours. The hybridization methods were similar to those described previously.19 Briefly, the DNA was denatured by immersion of the slides in 70% formamide 2X SCC at 70°C. After denaturing, the slides were dehydrated sequentially in 70%, 80%, and 95% ethanol and then air dried. The 70% ethanol was ice chilled. The chromosome 17-specific probe was a biotin-labeled α-satellite DNA probe (Oncor, Gaithersburg, MD). The hybridization mix consisted of 2 μg/mL probe DNA, 50% formamide in 2X SCC, and 500 μg/mL of carrier salmon sperm DNA. The probe mixture was denatured by heating to 70 to 75°C for 10 minutes. It was then cooled quickly on ice. After application of the denatured probe to the slides at 2 to 3 μL/cm², a coverslip was added and sealed with rubber cement. The slides were then incubated in a moist chamber for 12 to 16 hours at 37°C. After hybridization, the slides were washed at 47 to 49°C in 50% formamide 2X SCC (pH 7.0) for 30 minutes, placed in 1X SSC for 30 minutes, and then stored in 4X SSC (room temperature). Hybridized probe was detected with fluorescein-labeled avidin (Vector Laboratories, Burlingame, CA). In some cases propidium iodide (1 μg/mL) was used as a nuclear counterstain. The specimens were viewed at 100× magnification on a Reichert Microstar IV microscope (Cambridge Instruments Inc, Buffalo, NY) that was equipped for epifluorescence optics. Hybridization signals were enumerated for each of 200 to 1,000 cells. Photographs were taken with Kodak Ektachrome 400 color film (ASA 400) (Eastman Kodak, Rochester, NY), and the average exposure time was 1 to 2 minutes.

For correlative interphase studies routine smears of the bone marrow aspirate were used. The slides were used within 1 or 2 days of the aspiration or were frozen at −70°C and used at a later time (2 to 3 weeks). The slides were fixed with absolute methanol and then stained with Wright’s stain. Coverslips were applied with a dilute xylene:mounting medium (Pro-Texx; American Scientific, McGaw Park, IL) mixture. The slides were studied by routine light microscopy, and photomicrographs of cells suspicious for lymphoblasts were taken. Because we found that the hybridization could be more easily interpreted in areas where cells were not highly crowded, we attempted to photograph from more dilutely smeared areas of the slides. A microscope graduated stage was used to note the location of each cell photographed. After the photomicroscopy, the coverslips were carefully removed with incubation in xylene (5 minutes), and the slides were air dried. Without destaining or other pretreatment the slides were then hybridized as described previously. The hybridized probe was detected with fluorescein-labeled avidin as described above. Using the same microscope, the cells previously photographed were located and examined by fluorescent microscopy. The photomicrographs of the Wright-stained cells were projected in the vicinity of the fluorescent microscope so that simultaneous viewing of the previous light microscopic findings and the fluorescent hybridization results of the same cells was possible. Hybridization signals were enumerated for each cell.

RESULTS

The repeat bone marrow aspiration was performed within 1 week of the suspicious aspirate in the first patient and within 1 month in the second. The repeat aspirate from each patient showed a persistence of the immature lymphoid cells. In both patients the questionable cells increased slightly, from 7.5% to 10.6% of the total nucleated marrow cells in patient 1, and from 8.5% to 9.5% in patient 2 (Table 1). Immunologic and cytochemical studies were repeated on the specimen from the first patient, and the cells were found to be myeloperoxidase negative, and CD10 and CD19 positive as noted initially. Immunophenotyping studies were not performed on the specimen from the second patient, nor was TdT analysis repeated.

Interphase cytogenetics. In the in situ hybridization studies of lysed, hypotonically treated and fixed bone marrow cells (ie, without correlation to cell morphology), analysis showed that in patient 1, 106 of 1,000 interphase cells had three hybridization signals, indicating trisomy for chromosome 17. (In a separate hybridization with probes to both chromosome 17 and 9, the cells that showed three signals for chromosome 17 exhibited only two signals for chromosome 9; this excluded the possibility that the cells were triploid.) In patient 2, only 5 of 1,000 cells exhibited three signals, and this was within the range of three normal specimens where three signals were seen in 2 ± 3 of 1,000 cells (mean and 2 SDs) (Table 1).

For correlative interphase cytogenetic analysis, photographs of 24 suspicious cells in patient 1 and 23 cells in patient 2 were taken. After in situ hybridization, fluorescent signals could be enumerated in 18 of the cells from patient 1 and all of the cells from patient 2. Thirteen of the 18 cells in the first case (72%) (Fig 2A) and none (0%) of the cells in the second case (Fig 2B) exhibited three hybridization signals. The remainder of the evaluable cells photographed in patient 1 showed two signals.

Conventional cytogenetic analysis. The results of cytogenetic analysis are summarized in Table 1. At the end of therapy, 3 of 22 metaphase cells from the bone marrow of patient 1 had chromosomal abnormalities that represented karyotypic evolution of the original clone. Each of these three cells had an extra copy of chromosome 17. A normal female karyotype was noted in the posttreatment marrow sample from patient 2.

Follow-up. Patient 1, in whom residual or recurrent leukemia was demonstrated, returned for a follow-up bone marrow in 2½ months. The repeat study showed an overt relapse. Blasts accounted for 58% of the bone marrow cells in this specimen and cytogenetic analysis revealed karyotypic evolution (see Table 1). Interphase analysis showed three hybridization signals in 43% of 300 cells of this marrow. The patient was reinduced, but despite the treatment she died due to infectious complications and refractory disease.

Patient 2 had a follow-up bone marrow aspiration 3 months after the second study demonstrated normal cytogenetic findings. Morphologically suspicious cells persisted and accounted for 6% of the nucleated marrow cells. Conventional cytogenetic analysis showed normal chromosomes and no cells (0 of 20) with trisomy for chromosome 17 were detected when studied by interphase analysis. This patient continues in remission at 12 months after the completion of therapy.

DISCUSSION

Immature cells in bone marrow aspirates from patients treated for acute leukemia can present a diagnostic problem. In acute myelogenous leukemia (AML), neoplastic myeloblasts present in small numbers can often not be
Table 1.

<table>
<thead>
<tr>
<th>Clinical Course</th>
<th>Pathology</th>
<th>Cytogenetics</th>
<th>Interphase Cytogenetics (chr. 17)</th>
<th>Correlative Interphase Cytogenetics (chr. 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATIENT 1</td>
<td>Diagnosis</td>
<td>7/87 ALL-L1 92% blasts</td>
<td>46,XX(33%)/54,XX,+X, +X,+6,+14,+17,+18,+21, +22 (67%) (30 cells)</td>
<td>65% with 3 signals (200 cells) NA</td>
</tr>
<tr>
<td>Therapy</td>
<td>End-therapy 7/87-10/89</td>
<td>Lymphoid-morphology blasts (7.5%)</td>
<td>NA</td>
<td>NA NA</td>
</tr>
<tr>
<td>Follow-up</td>
<td>10/89</td>
<td>Lymphoid-morphology blasts (10.6%)</td>
<td>46,XX(86%)/52,XX,+6, +14,+17,+18,+21, +22(9%)/NCA: 55,XX,+X,+2,+6, +14,+16,+17,+18,+21, +22(5%) (22 cells)</td>
<td>10.6% with 3 signals (1,000 cells) 13/18 (72%) Lymphoid-morphology blasts with 3 signals</td>
</tr>
<tr>
<td>Relapse</td>
<td>1/90</td>
<td>Overt relapse (68% blasts)</td>
<td>46,XX(60%)/52,XX,+6,+14, +17,+18,+21,+22(30%)/52,XX,+6,+14,+17,+18,+21, +22, dup(1)(q12-q32) (10%)(20 cells)</td>
<td>43% with 3 signals (300 cells) ND</td>
</tr>
<tr>
<td>PATIENT 2</td>
<td>Diagnosis</td>
<td>9/87 ALL-L1</td>
<td>46,XX(58%)/55,XX,+X, +4,+6,+10,+14,+17,+18, +21, +21(38%)/NCA: 46,XX,t(11;16)(q13;q23) (4%) (24 cells)</td>
<td>NA NA</td>
</tr>
<tr>
<td>Therapy</td>
<td>End-therapy 9/87-1/90</td>
<td>Lymphoid-morphology blasts (8.5%)</td>
<td>NA</td>
<td>NA NA</td>
</tr>
<tr>
<td>Follow-up</td>
<td>2/90</td>
<td>Lymphoid-morphology blasts (9.5%)</td>
<td>46,XX(100%) (21 cells)</td>
<td>0.5% with 3 signals* (1,000 cells) 0/23 (0%) Lymphoid-morphology blasts with 3 signals</td>
</tr>
<tr>
<td>Remission</td>
<td>4/90</td>
<td>Lymphoid-morphology blasts (6%)</td>
<td>46,XX(100%) (23 cells)</td>
<td>ND 0/20 (0%) Lymphoid-morphology blasts with 3 signals</td>
</tr>
</tbody>
</table>

Abbreviations: NCA, nonclonal abnormality; (no. cells), number of cells analyzed; NA, not available; ND, not done.

*Within range of three control specimens in which three signals were seen in 0.2% ± 0.3% of cells (mean ± 2 standard deviations).

distinguished morphologically from regenerating blasts after myelosuppressive therapy. In ALL in pediatric patients the finding of immature cells with lymphoid morphology can pose diagnostic difficulties. Neoplastic lymphoblasts, especially those of L-1 morphology, may be morphologically similar to immature lymphoid cells (hematogones), which have been reported to be present normally in the marrow of children. These latter cells may not only be indistinguishable morphologically from malignant lymphoblasts, but they can also be similar immunophenotypically and with regard to TdT activity. Hematogones can express CD10, they can be TdT positive, and they can express surface markers of immature lymphocytes. Because hematogones have also been reported to be elevated in some children after chemotherapy, it has been suggested that to differentiate neoplastic lymphoblasts from hematogones, it may be necessary to correlate morphology, immunophenotype, and TdT reactivity with cytogenetic findings before a final diagnosis can be rendered.

In this report we describe two patients in whom suspicious cells presented a diagnostic problem; ie, were they hematogones or did they represent recurrent or residual leukemia? Because the problem arose at the end of therapy, its significance was more than academic; a decision about continuing therapy had to be made. Morphologic evaluation was not conclusive in assessing the nature of the blasts, and TdT reactivity and immunophenotyping were of little help. Because both patients had cytogenetic abnormalities at initial diagnosis, repeat bone marrow examinations were performed in hopes that cytogenetic findings could resolve the diagnostic problem. In addition to conventional cytogenetic study, the repeat aspirates were submitted for interphase cytogenetic analysis. These cases were well suited for this latter type of analysis because the original leukemic clone in each patient had numerical chromosomal abnormalities (ie, extra copies of multiple chromosomes). Interphase cytogenetic analysis can easily be used to detect trisomy for a targeted chromosome through in situ hybridization with an appropriate DNA probe.
and concluded that the suspicious cells were more likely to be hematogones. We could not exclude the possibility that the neoplastic cells had lost the extra copy of chromosome 17, but felt that this was unlikely, because it is more common for a leukemic karyotype to undergo clonal evolution with the development of additional karyotypic changes rather than to lose abnormalities noted initially. The additional finding in this case that there was no population of cells trisomic for the X chromosome (data not presented) also supported our conclusion because trisomy for the X chromosome had also been seen in the initial clone.

Furthermore, by performing the in situ hybridization on previously stained slides, we were able to correlate directly the cytogenetic findings with cell morphology. We demonstrated that the trisomy occurred specifically in the immature cells of question in the first patient, and not in the suspicious cells in the second. Therefore, the technique of interphase analysis on previously stained slides allowed for the morphologic identification of suspicious cells, and then for the subsequent determination of whether such cells carried a targeted cytogenetic abnormality.

Correlation of cytogenetic findings with cell morphology as illustrated here has a number of potentially important uses: it can be useful in the detection of residual leukemia, and it can be employed in the study of cell lineage and clonality in myelodysplasia and acute leukemia. In the detection of residual disease, correlative interphase cytogenetic analysis should increase the sensitivity of identifying
rare neoplastic cells. Our first case (patient 1) clearly illustrates its advantage over morphologic analysis alone. Morphologically suspicious cells could not be interpreted unequivocally as benign or malignant, but with the added information from interphase study the neoplastic cells could be positively identified. Although in this case conventional cytogenetic analysis and interphase analysis without morphologic correlation each detected the residual/recurrent disease, we suspect that when the neoplastic cells are fewer in number, the correlative approach should offer more sensitivity for the detection of rare malignant cells. For example, when residual/recurrent blasts are fewer than 1% or 2%, conventional cytogenetic study would require the analysis of more than 100 metaphase spreads to detect such a population, and noncorrelative interphase study is close to its limit of detection. The correlative interphase approach should increase the sensitivity of detecting rare malignant cells because it focuses the analysis on the cells in question and obviates the need of including in the analysis marrow elements that are obviously benign (eg, most erythroid and granulocytic precursors in a patient with ALL).

Correlative interphase cytogenetic analysis will clearly not be as sensitive as the polymerase chain reaction (PCR) in detecting rare neoplastic cells. The latter technique can be used to detect cells as infrequent as 1 in 10^4 to 10^5,19 Nevertheless, correlative interphase cytogenetic analysis is simple to use, easy to interpret, and more widely applicable than PCR. For example, numerical abnormalities in hyperdiploid ALL and in AML can be detected, and given new approaches to detecting structural chromosomal abnormalities in interphase cells, the applicability of this technology will become even broader.20

Correlative interphase cytogenetic analysis should also have important uses in the study of cell lineage and of the clonal origin of cells in acute leukemia and myelodysplastic syndromes. Through the analysis of cases previously demonstrated to have cytogenetic abnormalities suitable for interphase study, it will be possible to determine quickly and easily which cell lines are involved in the neoplastic clone, and to what extent the involvement exists. With the development of new treatment strategies in myelodysplasia and leukemia (eg, growth factor therapy), it may become useful to be able to define the lineage involved in the neoplastic condition and to monitor the involvement throughout therapy.21,22

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