

# Interphase Cytogenetic Analysis Detects Minimal Residual Disease in a Case of Acute Lymphoblastic Leukemia and Resolves the Question of Origin of Relapse After Allogeneic Bone Marrow Transplantation

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We used *in situ* hybridization with a probe for the X chromosome to study interphase cells of bone marrow and peripheral blood specimens from a male patient with acute lymphoblastic leukemia characterized by hyperdiploidy, including trisomy X. In a posttreatment bone marrow specimen, which was interpreted as a regenerating bone marrow morphologically and which demonstrated a normal karyotype cytogenetically, trisomy X was found in 16 of 1,000 interphase cells. This finding indicated the presence of leukemic cells that were undetected by conventional morphologic and cytogenetic techniques (ie, minimal residual disease). Cytogenetic studies of a relapse specimen obtained after a sex-mismatched bone marrow transplant showed only a normal

female karyotype in each of 40 metaphase cells, suggesting that the relapse occurred in donor cells. However, interphase analysis demonstrated trisomy X in more than 80% of interphase cells and indicated that the relapse was of the original clone and was not a transformation of donor cells. This case illustrates that interphase analysis can be useful as an adjunct to conventional cytogenetic analysis in the detection of minimal residual disease and in the analysis of interphase cells that are not accessible to routine cytogenetic methods. It also illustrates that previously reported instances of relapse of leukemia in donor cells could have been incorrect if supported by cytogenetic data alone.

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**A**DVANCES IN CYTOGENETIC and molecular techniques have made it possible to quickly and easily detect numerical chromosomal abnormalities in interphase nuclei.<sup>1-4</sup> By hybridization of a chromosome-specific DNA probe to interphase cells, and by detection of the hybridized probe with methods that do not require the use of radioisotopes, it is now possible to determine the copy number of a targeted chromosome by simply enumerating hybridization signals. Although this interphase analysis provides only limited information about the karyotype, when it is used in conjunction with conventional cytogenetic study, it has specific advantages and particular applications that make it useful both in the management of patients with hematologic malignancies and in the study of leukemic cells. For example, because the technique can be used for the study of nondividing or slowly dividing cells, it can be used for studies of terminally differentiated blood cells or of cells that are difficult to grow in culture. Because these methods can also be used for rapid screening of large numbers of cells, they can also be applied to the search for residual disease in patients in remission from acute leukemias previously shown to have numerical chromosomal abnormalities.

In this report, we describe the use of interphase cytogenetic analysis in the study of a case of acute lymphoblastic leukemia (ALL) characterized by a hyperdiploid karyotype and trisomy X. We used the technique to resolve the question of the origin of relapse after a sex-mismatched bone marrow transplant (BMT). Data from conventional cytogenetic analysis suggested a relapse in donor cells, whereas interphase study demonstrated that the original leukemic clone had recurred. Furthermore, we used the technique to detect minimal residual disease in a specimen that, on morphologic and karyotypic analysis, showed no residual leukemia.

## CASE PRESENTATION

The patient was a 23-year-old man who was diagnosed with ALL in March 1987 (see Table 1). The initial diagnostic BM specimen showed that the hematopoietic space was packed with lymphoblasts with L-1 morphology. The blasts accounted for more than

90% of the BM cells and were immunophenotyped as precursor B cells. Conventional cytogenetic analysis of unstimulated BM and peripheral blood (PB) cells cultured for 24 hours showed an abnormal hyperdiploid male karyotype (60 chromosomes) that was characterized by a gain of the X chromosome (two copies) and of chromosomes 4, 6, 7, 8, 10, 12, 14 (two copies), 17, 18, and 21 (two copies), and by an unbalanced translocation involving chromosome 7 (Table 1).

The patient was treated on a standard protocol (Cancer and Leukemia Group B No. 8513) and received systemic and intrathecal chemotherapy. In response to this treatment, he entered a remission within 3 months. The remission BM specimen on day 61 of therapy (June 1987) was interpreted on pathologic examination as a regenerating marrow with no evidence of residual leukemia. Although blasts were increased and accounted for 8% of the nucleated BM cells, they appeared to be myeloblasts and part of the regenerative process. Cytogenetic analysis of this specimen confirmed the impression of remission, as each of 20 metaphase cells examined showed a normal male karyotype.

Six months later (January 1988), after further consolidation therapy, a routine follow-up BM biopsy showed a relapse. The BM marrow aspirate showed 23% blasts, and these had a morphologic appearance similar to those noted at initial diagnosis. Intensified chemo-radiotherapy was administered, and the patient underwent allogeneic BMT with his sister as donor.

After the transplant, the patient did well and had evidence of

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Table 1.

Date	Clinical Course	Pathology	Cytogenetics†	Interphase Cytogenetics * (X chromosome)
3/87	Diagnosis induction	ALL L1 100% cellular 92% blasts	46,XY(81%)/60,XY,+X,+X,+4,+6, +7,+8,+10,+12,+14,+14,+17, +18,+21,+21,der(7)t(1;7)(q21;q36) (14%)/NCA:59,XY,+X,+X, +4,+6,+7,+10,+12,+14,+14, +17,+18,+21,+21(3%)/52,XY, +7,+9,+11,+12,+18,+22,del(7) (p15p22)(3%) (36 cells)	NA
4/87	Repeat induction	Residual ALL 5% cellular 90% blasts	ND	ND
6/87	Remission	Regenerating BM 40% cellular 8% blasts	46,XY(100%) (20 cells)	1.6% trisomy X (1,000 cells)
9/87	Remission intensification	Rare blasts 35% cellular 2% blasts	ND	0% trisomy X (1,000 cells)
10/87	Remission intensification	Granulocytic hyperplasia 75% cellular 1% blasts	ND	0% trisomy X (2,000 cells)
1/88	Relapse reinduction	Recurrent ALL 50% cellular 23% blasts	46,XY(100%) (30 cells)	NA
3/88	TBI/Cy BMT (sister)	NA	NA	NA
6/88	Remission	Rare blasts 20% cellular 1% blasts	46,XX(100%) (20 cells)	NA
9/88	Relapse	Recurrent ALL 100% cellular 98% blasts	46,XX(100%) (40 cells)	BM*:82.7% trisomy X (1,000 cells) PB:17.0% trisomy X (1,000 cells)
3/89	Expired			

Abbreviations: NA, not available; ND, not done; NCA, nonclonal abnormality; TBI/Cy, total body irradiation/cyclophosphamide.

\*Trisomy 17 was also detected in 75% of the cells of this specimen by in situ hybridization.

†Cytogenetic analyses from 3/87 and 9/88 were performed on BM and PB cells. All other analyses were performed on BM cells.

engraftment, with recovery of his PB counts. At 3 months posttransplant, all marrow metaphase cells were female. However, despite the success of the transplant the patient relapsed again in September 1988. The posttransplant relapse specimen (Fig 1) was similar in appearance to the initial diagnostic BM specimen. Blasts accounted for 98% of the nucleated BM cells. Cytogenetic analysis of peripheral blood and BM cells cultured for 24 hours showed a normal female karyotype in each of 40 metaphase cells examined. Because blasts accounted for nearly all of the marrow cells, this cytogenetic finding led to the speculation that the posttransplant relapse had occurred in donor cells.

#### MATERIALS AND METHODS

BM cells prepared for the routine cytogenetic studies were used for interphase analysis. The cells had been cultured for 24 hours without mitogens, treated with Colcemid (GIBCO, Grand Island, NY) for 10 to 25 minutes (0.05 µg/mL), incubated with hypotonic KCl (0.075 mol/L), and then fixed with absolute methanol:glacial acetic acid (3:1 vol/vol). We made air-dried preparations by dropping the cell suspensions onto glass slides; the slides were then dried in an oven for 2 hours at 65°C. The cells were hybridized with a probe to the  $\alpha$ -satellite region of the X chromosome; the probe had been nick-translated with biotinylated dUTP (Bio-dUTP; Oncor Inc, Gaithersburg, MD). The hybridization procedure was

similar to that reported earlier.<sup>5</sup> The final hybridization mix consisted of 2 µg/mL probe DNA, 50% formamide, 2X SSC, and 500 µg/mL of salmon sperm DNA. The hybridized probe was detected with fluoresceinated avidin, and through amplification with a second layer of fluoresceinated avidin after treatment with biotinylated goat anti-avidin antibodies. The nuclei were counterstained with propidium iodide (1 µg/mL) in an antifade solution.

Control specimens consisted of four BM samples obtained from male patients undergoing autologous BMT for nonhematopoietic malignancies. All specimens were analyzed by routine fluorescence microscopy. One thousand to 2,000 interphase nuclei were analyzed from each specimen studied, and the number of hybridization signals per nucleus was noted. The rare metaphase cells were also counted and scored. Photographs were taken on an epifluorescence microscope equipped for photography. Kodak Ektachrome film 400 ASA (Eastman Kodak Co, Rochester, NY) was used and the average exposure time was 1 to 2 minutes.

#### RESULTS

The BM and PB specimens obtained at the time of relapse after BMT (September 1988) and studied by interphase analysis showed a high percentage of cells with three hybridization signals, indicating trisomy for the X



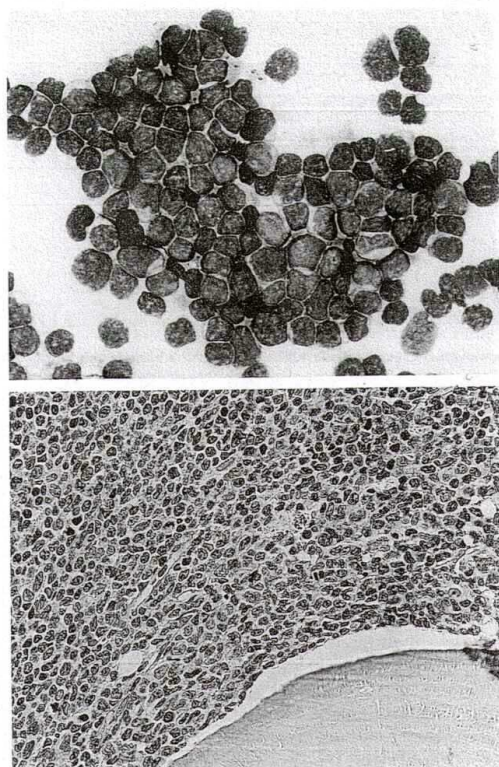


Fig 1. BM touch preparation and biopsy showing the posttransplant relapse of ALL. Almost all of the cells are lymphoblasts; only rare granulocytes are present.

chromosome. In the BM, trisomic cells accounted for 82.7% of the 1,000 nuclei analyzed (Table 1 and Fig 2A). Cells with two hybridization signals, indicating two copies of the X chromosome, accounted for 16.6% of the cells in this specimen and nearly one-half of these appeared to be segmented neutrophils. Only rare (0.5%) cells had a single

hybridization signal. Interphase analysis of PB cells showed that 17.0% of the cells had three hybridization signals. All of the segmented nuclei in the PB exhibited only two signals (Fig 2B). The rare metaphase cells encountered in each of these specimens (mitotic rate = 0.25%) had only two hybridization signals and this was consistent with the results of routine cytogenetic analysis in which only metaphase cells with a normal female karyotype was observed.

Interphase study of the "remission" specimen of June 1987, before the transplant, showed that most of the nuclei had a single hybridization signal, indicating the normal complement of one X chromosome in a male (Fig 3A). However, 16 of 1,000 nuclei clearly showed three signals, indicating trisomy X (Fig 3B). Of the total of 4,000 cells from the four normal male control specimens, only one cell showed three signals. Thus, the finding of 16 of 1,000 cells with three signals was highly significant ( $P < .01$  by two-tailed  $\chi^2$  analysis).

The remission specimens from September 1987 and October 1987 showed no cells with three signals. Each of the 29 metaphase cells analyzed from the remission samples (mitotic rate 0.9%) showed only one or two hybridization signals (in 24 and 5 cells, respectively).

#### DISCUSSION

After an allogeneic BMT of cells from his sister, the patient described here had a relapse in which the dividing cells from a BM sample exhibited a normal female karyotype. Because almost 100% of the BM cells were lymphoblasts, and because each of the 40 mitotic cells analyzed demonstrated this karyotype, it was reasonable to speculate that the case was a rare example of leukemia relapsing in donor cells. Relapse of leukemia in donor cells has been reported in 10 published cases.<sup>6-15</sup> The cases have included relapses of acute and chronic leukemias and relapses occurring early and late after the transplantation. One of the hypotheses proposed for the mechanism of such relapses is that a transmissible agent or environmental factor might first transform the host cells and then persist, and later transform the donor cells as well.

In an attempt to verify the suspicion of relapse in donor cells in our case, we used interphase cytogenetic analysis

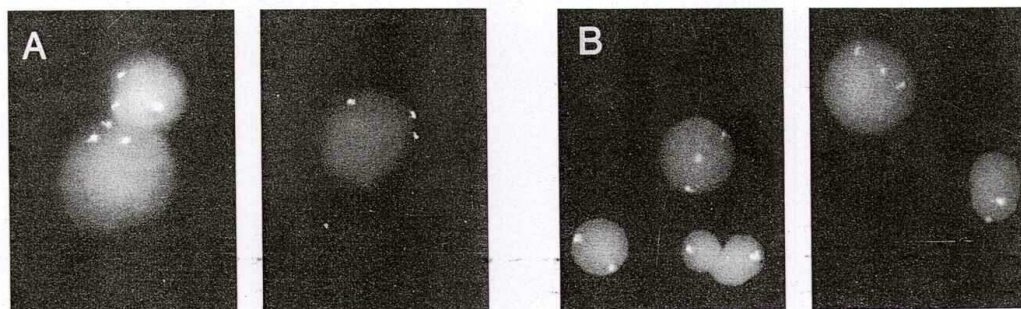


Fig 2. Specimens from the posttransplant relapse BM (A) and PB (B) hybridized with a probe for the X chromosome. Nuclei with three hybridization signals are seen in both specimens. The PB sample also shows two signals in nuclei of segmented granulocytes.



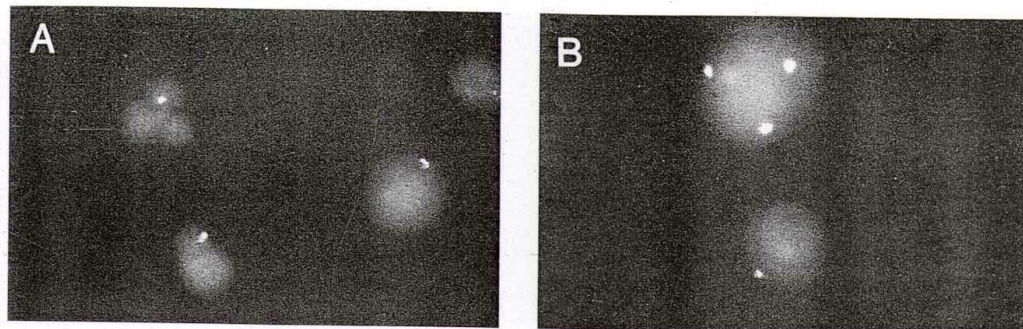


Fig 3. Specimens from the June 1988 "remission" BM hybridized with a probe for the X chromosome. Most cells exhibit a single hybridization signal indicating a normal complement of one X chromosome in a male (A). Rare cells (B) showed three signals indicating trisomy X and residual leukemia.

with a probe specific for the X chromosome. We reasoned that interphase analysis with an X-specific probe would allow us to analyze all cells and to discriminate among normal male cells (one hybridization signal), normal female cells (two hybridization signals), and the cells of this patient's original leukemic clone (trisomy X, three hybridization signals). Our finding of three hybridization signals (trisomy X), in more than 80% of the BM cells indicated that the relapsed leukemia was a recurrence of the original disease and not a relapse in donor cells. Supporting this conclusion were additional findings: Y bodies, as determined by Y-body fluorescence with quinacrine dihydrochloride, were detected in 85% of cells of the relapsed specimen and trisomy 17 (also present in the initial clone) was detected in 75% of the relapse cells by *in situ* hybridization. Clearly, the relapse was not 46,XX as suggested by metaphase analysis.

The reason for our inability to detect the original clone at relapse by conventional cytogenetic analysis is not clear, but may be related to the kinetics of the neoplastic cells. Although blasts filled the BM and greatly outnumbered the donor myeloid cells, the mitotic activity of the neoplastic lymphoblasts may have been significantly lower than that of the nonmalignant elements. It has been demonstrated that the rate of proliferation of the leukemic cells in some cases of acute leukemia is significantly lower than that of normal marrow cells, and that expansion of the neoplasia occurs because of gradual accumulation of the neoplastic cells and their lack of differentiation.<sup>16</sup> Thus, the inability to detect mitoses of the recurrent disease may have been attributable to a very low mitotic rate of the neoplastic cells.

Alternatively, the proliferative activity of the leukemic cells may have been suppressed in culture. Keinanen et al have recently shown by use of the morphology, antibody, chromosomes (MAC) technique that the relative proportion of mitoses of different types of BM cells is not maintained in culture.<sup>17</sup> The number of mitoses of erythroid cells was found to decrease precipitously to zero within 24 hours, whereas the number of granulocyte mitoses increased. Perhaps, in the present case, the mitotic rate of the host leukemic cells decreased to a point where they could

not be detected by conventional metaphase analysis, whereas the mitotic rate of the donor myeloid cells increased.

Whether it was because there were too few mitoses for detection or because mitotic activity of the neoplastic cells was suppressed in culture, we were unable to detect the recurrent leukemia in this case by conventional cytogenetic analysis. It seems possible that a similar inability to detect recurrent disease by conventional cytogenetic study may have led to an interpretation of relapse in donor cells in some of the previously reported cases. In eight of the 10 published cases, the notion of relapse in donor cells was supported by conventional cytogenetic data alone (including the use of chromosomal variants in two cases), and in five of these cases, the karyotype of the relapse specimen showed only normal donor cells. In an additional recent case, cytogenetic analysis suggested relapse in donor cells, but molecular studies demonstrated the recipient's DNA in the majority of the BM cells.<sup>18</sup> The authors of this report felt that the origin of the relapse was unresolved; however, they questioned whether the analysis of metaphase cells may have detected only residual donor cells and was blind to the recurrent neoplasia. Data from the present case suggests that metaphase analysis may very well have been unable to detect the recurrent disease.

Identification of the origin of the relapsed leukemia in this case illustrates an important advantage of interphase cytogenetic analysis over conventional study; ie, the interphase method is not dependent on proliferative activity or on the presence of metaphase cells. The detection of trisomic cells, ie, of minimal residual disease, in the specimen considered to be a remission specimen demonstrates a second advantage; that is, by means of interphase cytogenetics one can detect a low frequency abnormality. The specimen from June 1987 showed no karyotypically abnormal cells in 20 metaphases analyzed. However, by interphase analysis we detected abnormal cells at a frequency of 1.6%. Statistically, 114 to 148 metaphases would have to be analyzed for detection of such a rare population at a confidence level of 90%.

The sensitivity of interphase analysis in the detection of the targeted chromosomal abnormality in this case deserves



some comment. The rate of false trisomy, as determined from the study of the four control specimens, was extremely low (1 in 4,000 or 0.025%). This result may be because of the fact that three signals cannot easily be formed artifactually in a normal male cell. Detection of trisomy for autosomes, or for the X chromosome in females, appears to be somewhat less sensitive. We have determined that the false-trisomy rate for chromosome 17 in normal marrows is  $0.2\% \pm 0.3\%$  (mean  $\pm$  2 SD, data not presented); this is in agreement with the findings of Giwerzman et al, who reported false trisomy for chromosome 1 in seminal fluid specimens to be 0.2%.<sup>19</sup> The cause of the false trisomy cells is not readily apparent, but it may be artifactual splitting of one signal, replication of one centromere before the other, or overlapping of two signals in a tetraploid cell. Detection of monosomy of a targeted chromosome by interphase study is less sensitive than the detection of trisomy.

Although false positivity in interphase cytogenetic analysis needs further study, the use of interphase technology clearly offers an important adjunct to conventional cytogenetic analysis in the study of malignancies with numerical chromosomal abnormalities. In particular, a complete cytogenetic analysis could be performed at the time of diagnosis. Thereafter, interphase cytogenetic analysis could be used for monitoring of patients using the appropriate probes indicated by the initial conventional cytogenetic work-up. With the development of technology to detect structural chromosomal anomalies in interphase cells, interphase analysis will have even wider application.

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