Origin of Leukemic Relapse After Bone Marrow Transplantation: Comparison of Cytogenetic and Molecular Analyses


Bone marrow transplantation (BMT) is the treatment of choice for many patients with poor prognosis, relapsed, or refractory leukemia who have matched donors. Despite the aggressive nature of this therapy, malignancy recurs in approximately 30% to 50% of patients who undergo allogeneic BMT for treatment of acute leukemia. Most instances of post-BMT leukemia probably represent regrowth of the original leukemic clone. However, relapse in donor lineage cells has been reported in acute and allogeneic BMT for treatment of acute leukemia. Most observed in derived leukemia may account for up to 5% of all leukemias. It has been estimated donor-derived cells have also been described following allogeneic BMT occurring in 30% to 50% of patients who undergo relapse in approximately or refractory leukemia who have matched donors.

Analyzing each case is important for our understanding of leukemogenesis and the biology of BMT. Blood, cytogenetic polymorphisms. Molecular biological tools provide a new and powerful approach to the study of post-BMT engraftment and leukemic relapse. This approach utilizes DNA restriction fragment length polymorphisms (RFLPs) in the human genome to distinguish donor and recipient cells. Since some DNA loci are highly polymorphic, the use of only a small number of restriction enzymes and a handful of probes is sufficient to distinguish donor- and recipient-derived cells in 99% of BMT patients.

In this study, a case of post-BMT relapse was analyzed using both cytogenetic and molecular techniques. Each type of analysis resulted in an apparently unambiguous assignment of the lineage of the leukemic cells. However, the results of the two analyses did not agree. Cytogenetic studies indicated that the leukemic relapse occurred in donor cells while molecular studies indicated that it occurred in the recipient's own cells. These results indicate the need for a broad-based assessment of engraftment status that will be less susceptible to the technical limitations of any given method. In addition, they suggest that some apparently clear cases of donor cell transformation in bone marrow recipients may not be substantiated on closer scrutiny.

Bone marrow transplantation (BMT) is generally due to the recurrence in recipient cells, but may rarely occur as a result of donor cell transformation. Donor cell relapse is generally identified using cytogenetic markers such as the sex chromosomes. Recently, molecular techniques have been used to identify the origin of bone marrow cells by their DNA restriction fragment length polymorphisms. We describe the case of a male pediatric patient who had a leukemic relapse 30 months following BMT from his sister. Both cytogenetic and molecular techniques were used to identify the origin of the leukemic relapse. Cytogenetic analyses indicated the absence of the Y chromosome and the presence of a donor cell type 9qh polymorphism, suggesting a donor cell relapse. Molecular analyses also indicated the absence of the Y chromosome but demonstrated the recurrence of recipient DNA markers from three other chromosomes, suggesting a recipient cell relapse. While the leukemic cell lineage cannot be definitively assigned in this case, our results suggest that caution must be exercised when assigning leukemic cell lineage following post-BMT relapse.

**CASE REPORT**

In December 1982, a 2-year-old male child with profound anemia, thrombocytopenia, and hepatosplenomegaly was found to have acute lymphoblastic leukemia (ALL). His WBC count was 9.2/10³ µL with 39% circulating blast forms. Bone marrow aspiration revealed near total marrow replacement with lymphoblasts that were predominantly of FAB L2 morphology. Neither surface immunoglobulin nor T cell-specific antigens were present on blast cells. Remission was successfully induced using a combination of vincristine and prednisone. An isolated CNS relapse in September was successfully treated with intrathecal chemotherapy. Remission in both the CNS and bone marrow occurred by December 1983.

At this time, the patient was referred to the Pediatric Hematology/Oncology service of Rainbow Babies and Children's Hospital, Cleveland. The diagnosis of ALL was confirmed by examination of peripheral blood, bone marrow, and CSF. Bone marrow aspiration revealed 79% blast forms. When bone marrow cells were tested for surface antigens, 58% expressed CALLA (CD10) and 62% expressed OK Ia-1 (monomorphic HLA Dr). Remission was successfully induced using a combination of vincristine, prednisone, l-asparaginase, daunorubicin, and intrathecal methotrexate. In February 1984, the patient was enrolled in an institutional study for the treatment of relapsed leukemia. The protocol involves bone marrow ablation with high-dose cytosine arabinoside (ara-c) and total body irradiation (TBI) followed by allogeneic BMT. The patient received bone marrow from his HLA-A, -B, and -C identical and mixed lymphocyte culture (MLC) nonreactive sister. He developed mild graft-

From the Departments of Pediatrics and Biology, Case Western Reserve University, Cleveland; the Department of Pediatrics, University of Nebraska Medical Center, Omaha; the Department of Human Genetics, Collaborative Research, Inc., Bedford, MA; and the Department of Pediatrics and the Institute of Human Genetics, University of Minnesota Medical School, Minneapolis.

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Address reprint requests to Naomi Lang-Unnasch, PhD, University of Alabama at Birmingham, Division of Geographic Medicine, 1025 18th St South, Birmingham, AL 35205.

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versus-host-disease (GVHD) of the upper gastrointestinal (GI) tract that was demonstrated by biopsy and was resolved with corticosteroid treatment.

In July 1986, more than 800 days post-BMT, the patient developed facial ecchymosis and diffuse bone pain. Peripheral blood analysis revealed a WBC count of 10.7 x 10^3/L without circulating blast forms. Bone marrow aspiration revealed 30% lymphoblasts with morphology and cell surface markers identical to those observed in December 1983. The patient was again treated with a combination of vincristine, prednisone, l-asparaginase, and daunomycin. He achieved remission within 1 month and was placed on a maintenance chemotherapy regimen. In November, peripheral blood and bone marrow analyses documented continued remission status. The WBC count was 2.9 x 10^3/L and bone marrow aspirates contained only 1% blast forms. In January 1987, the patient again experienced ALL relapse. The WBC cell count was 5.4 x 10^3/L without identifiable circulating blast forms and bone marrow aspiration revealed 68% lymphoblasts. Administration of further chemotherapy resulted in pancytopenia and the patient died of sepsis in February 1987.

**MATERIALS AND METHODS**

**Cytogenetic analysis.** Stimulated peripheral blood cultures were initiated in RPMI 1640 (GIBCO, Grand Island, NY) with phytohemagglutinin (PHA) (GIBCO M-form). After incubation for 72 hours, colcemid was added for one hour. Each bone marrow culture was initiated in McCoy’s 5A and in RPMI 1640 media without PHA. A direct harvest was performed after 30 minutes in colcemid. Cells were also incubated for 24 hours and harvested after one hour of colcemid treatment.

All harvests were performed with 0.075 mol/L KCl. The cells were fixed in Carnoy’s fixative, and stained with Quinacrine or Giemsa.

**Molecular analysis.** High molecular weight (mol wt) DNA was prepared from the patient’s normal skin fibroblast, bone marrow, and peripheral blood, and from the donor’s peripheral blood. DNA was analyzed as described by Knowlton et al., unless otherwise indicated. The appropriate blots were probed with ^32P-labeled St14-1, pDP34, pDP132, pDP105, L4-427, L4-123, or S-232. All work with recombinant microorganisms was performed under P1 conditions of containment in accordance with National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

**RESULTS**

**Cytogenetic data.** The patient’s bone marrow and/or peripheral blood was karyotyped on two occasions prior to BMT and nine times post-transplantation (Table I). At the time that ALL was first diagnosed, the patient’s bone marrow cells had a karyotype of 46, XY, t(18;21) (Dr. H. Robinson, personal communication, February 1988). A pre-transplant karyotype analysis of a bone marrow sample taken in February 1984, after induction of remission, was normal 46, XY. One cell of the fifteen cells karyotyped was missing a Y chromosome. Pre-transplant analysis of peripheral blood from the donor indicated she had a normal 46, XX karyotype. In addition to the sex chromosome differences between the donor and patient, a G-band polymorphism in the heterochromatic region of chromosome number 9 (9qh) was demonstrated.

Bone marrow and stimulated peripheral blood samples taken from the patient 2 months and 1 year post-transplant revealed a normal 46, XX karyotype. At the time the patient was admitted with symptoms of leukemic relapse (July 1986), cytogenetic analysis again indicated the presence of donor cells with a normal 46, XX karyotype in the peripheral blood and bone marrow. The translocation observed in the patient’s bone marrow cells at initial diagnosis [t(18;21)] was not observed in this sample or in any of the samples analyzed following relapse. One cytogenetic abnormality that was observed was the presence of polyploidy in approximately 20% of the marrow cells. The poor quality of the polyploid metaphase spreads precluded karyotype analysis.

The bone marrow samples taken during remission, August 20, 1986 and November 19, 1986, contained few polyploid cells. Of the seven cells scored as polyploid on these dates, only one cell was of sufficient quality for karyotype analysis. This analysis demonstrated that the cell was 92, XXXY with no obvious chromosomal rearrangement. The chromosome 9qh polymorphism also matched that of the donor (Fig 1).

Following the leukemic relapse in January 1987, two further bone marrow samples were analyzed. The sample from January 12, 1987 contained 68% polyploid cells. It was possible to score 17 of these cells. All cells contained an abnormal tetraploid karyotype with multiple marker chromosomes. The only consistent chromosomal abnormality was the rearrangement of two copies of chromosome 6. Nine cells contained 4 X chromosomes, four contained 3 X chromosomes, and four contained 2 X chromosomes. There was no evidence of a Y chromosome in any of these spreads. Two cells containing 3 X chromosomes and one containing 2 X chromosomes were further analyzed. The 9qh polymorphism in these cells matched that of the donor (Fig 1). In addition to the rearrangement in two of the four copies of chromosome 6, a rearrangement in two of the four copies of chromosome 19 was present in one of these cells (Fig 2). In addition to the polyploid cells, 14 diploid cells from this sample were analyzed. The diploid cells showed a high rate of random chromosome loss, but had an otherwise normal 46, XX karyotype.

The karyotype of the diploid cells in the final bone marrow sample (January 28) was similar to that of January 12. One cell that had an XO karyotype was shown to have the donor’s 9qh polymorphism. There were fewer polyploid cells in this sample (6%), and their poor quality precluded karyotype analysis.

Y body fluorescence counts were also performed on all samples obtained since July 11, 1986 (Table 1). The mean percentage of Y bodies scored during this 6-month interval was 1.8%. Since Y body counts of normal male cells are generally 40%, these data corroborated the karyotype analysis that suggested the absence of the Y chromosome from bone marrow and peripheral blood cells.

**Molecular data.** A total of seven informative DNA probes were used to analyze a portion of the bone marrow samples used for the November 1986 (remission) and January 12, 1987 (relapse) cytogenetic analyses (Table 2). Five of the probes hybridize with DNA sequences on the sex chromosomes. The two probes that were informative only for Y chromosome-specific restriction fragments (pDP105 and pDP132) hybridized with DNA isolated from the fibroblast.
Table 1. Summary of Cytogenetic Analyses

| Date     | Remission Status | Bone Marrow (Unstimulated) | Peripheral Blood-Stimulated (PHA) Cultures | Total No. of Cells Karyotyped (#) | Predominant Diploid Karyotype (ff) | Percentage of Polyploid Cells | Percentage of Cells With Y-Bodies
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<td>-</td>
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Abbreviation: CR, complete remission; R, relapse.
*Pretransplant.
†Normal male values for Y-Body Counts: 40%.²⁵

cells of the patient, but not with DNA from either bone marrow sample (data not shown). In contrast, the X chromosome probes (St14-1 and S-232) indicated that the predominant cell lineage in the bone marrow dramatically changed during the interval between November 1986 and January 1987. Figure 3 shows that during the remission period in November, St14-1 recognized a set of restriction fragments identical to that of the donor. However, St14-1 detected both donor- and recipient-specific restriction fragments in DNA isolated from bone marrow cells taken following the January relapse. In this sample, which contained almost 70% lymphoblasts, the weak intensity of the donor-specific restriction fragments suggested that <10% of the DNA was of donor origin (Fig 3, lane 4). In addition, RFLP analysis of January peripheral blood cells also indicated the predominance of the patient's own restriction fragments.

One of the probes used, pDP34, hybridizes with both X- and Y-specific DNA sequences. When pDP34 was used to probe DNA from the two bone marrow samples, the results were qualitatively but not quantitatively identical. As shown in Fig 4, neither sample contained the Y chromosome-specific 15 kilobase (kb) Taq1 restriction fragment. This was consistent with the results obtained from the other Y chromosome probes. Both samples contained X chromosome-specific Taq1 fragments of 11 kb and 12 kb. In the November DNA sample and in the donor cell DNA, equal amounts of the 11 and 12 kb restriction fragments hybridized with pDP34. In the January DNA sample, much more of the

Fig 1. Pretransplant chromosomal polymorphisms between the donor (D) and the recipient (R) were used to identify the lineage of the patient's bone marrow cells following BMT. The polymorphic heterochromatic region of the recipient's chromosome 9 is indicated with an arrow. The post-transplant (PT) samples were obtained during remission (August 20, 1986, PT-A) and during relapse (January 12, 1987, PT-B). The karyotype of one tetraploid cell observed during remission had the donor cell 9qh polymorphism and had four X chromosomes indicating that it arose from a donor cell lineage (PT-A). In the BMT sample obtained during relapse, analysis of three near tetraploid cells with fewer than four X chromosomes indicated the presence of the donor cell 9qh polymorphism (PT-B).
11-kb than the 12-kb restriction fragment hybridized with pDP34. Since the patients fibroblast cell DNA hybridized with the 11-kb but not the 12-kb fragment, a mixture of donor and recipient cells would be expected to contain more of the 11-kb than the 12-kb fragment. The simplest interpretation of this result is that the January bone marrow sample contained a mixture of donor and recipient cells. This interpretation is consistent with that for the other X chromosome probes.

Since RFLP analyses with X chromosome probes contradicted the cytogenetic analyses, the two bone marrow DNAs were also analyzed with two informative probes for autosomal sequences (chromosomes 5 and 21). The restriction fragments detected by these probes also indicated the

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**Table 2. Summary of Molecular Analyses**

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<th>Probe</th>
<th>Enzyme</th>
<th>Chromosomal Locus</th>
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<td>21q22</td>
<td>Donor</td>
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</tr>
<tr>
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<td>TaqI</td>
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</tr>
<tr>
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<td>TaqI</td>
<td>Xq 21.31 &amp; Yp</td>
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</tr>
<tr>
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<td>S-232</td>
<td>TaqI</td>
<td>Xp22</td>
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**Bone Marrow**

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| Abbreviation: ND, not done.  
*See text for details.*
absence of recipient cells in the November bone marrow DNA sample and their predominance in the January sample (Fig 5).

DISCUSSION

The cytogenetic analyses of this case all support the conclusion that in vivo transformation of donor cells resulted in the post-BMT leukemic relapse. No Y chromosomes were seen post-transplant and there was no evidence of Y-body fluorescence in 7,000 interphase nuclei examined at various times following the July 1986 relapse. This indicates that recipient cells, if present, had a 45, XO karyotype. The predominant karyotype of 233 diploid cells examined following transplantation was that of the donor, 46, XX. Only three cells had a 45, XO karyotype and these contained the donor chromosome 9th polymorphism.

After the post-BMT relapse in July 1986, there was a marked increase in the fraction of diploid cells with random chromosome loss. However, there was no evidence for the reemergence of the chromosomal rearrangement noted at the original onset of ALL [t(18;21)]. There did appear to be a positive correlation between the percentage of polyploid cells and the percentage of lymphoblasts in the bone marrow. This suggests that the polyploid cells might represent the malignant clone. The polyploid cells that were analyzed from the January bone marrow sample were near tetraploid and contained unclassifiable chromosomal fragments. Most of the cells scored contained four X chromosomes and two identically rearranged chromosome 6s (6pq +). The simplest interpretation of these findings is that a transformed 46, XX donor cell with abnormalities including one rearranged chromosome 6 underwent non-disjunctive mitosis. The resulting tetraploid cell then became more cytogenetically abnormal as a result of further chromosomal rearrangements and of random losses or gains of individual chromosomes. Errors resulting in such chromosomal abnormalities commonly affect chromosomes in tumor cells.

In contrast to these conclusions, the molecular data demonstrate that at relapse, there was a dramatic replacement of donor-derived bone marrow cells by recipient cells. During remission, the DNA probes detected only the RFLPs of the donor cell DNA. At relapse, both donor and recipient RFLPs were detected, but at least 90% of this bone marrow cell DNA was from recipient cells. The DNA probes used indicated the presence of recipient DNA from two different autosomes and from both arms of the X chromosome. Only probes that hybridize with the Y chromosome did not detect the presence of recipient cell DNA. Y chromosome loss is known to occur in cases of leukemic and myeloproliferative disorders. It should be noted that it would be highly unlikely that a chromosomal rearrangement could alter even

![Fig 3. DNA polymorphisms detected by the probe St14-1. The donor-specific (D) and recipient-specific (R) restriction fragments identified in genomic DNA samples from peripheral blood of the donor (lane 1) and fibroblasts of the recipient (lane 2) are indicated at left. Post-BMT patient DNA was isolated from bone marrow (lanes 3 and 4) and peripheral blood (lane 5) samples obtained at two times. During remission, November 19, 1986, the DNA has all the donor-specific RFLPs but none of the recipient-specific RFLPs (lane 3). During relapse, January 12, 1987, the DNA has both donor- and recipient-specific RFLPs but recipient-specific RFLPs predominate (lanes 4 and 5). For this experiment, the filter was washed three times for 30 minutes each time at 55°C in 0.1 × SSC and 0.1% SDS (2 × SSC − 0.3 mol/L NaCl, 0.03 mol/L sodium citrate; SDS, sodium dodecyl sulfate).](image-url)

![Fig 4. DNA polymorphisms detected by the probe pDP34. DNA isolated from peripheral blood of the donor (D-747) has two X chromosome-specific restriction fragments of 11 and 12 kb. DNA isolated from recipient fibroblasts (R-774) has the 11-kb X chromosome-specific restriction fragment and a 15 kb Y chromosome-specific restriction fragment. Post-BMT patient DNA isolated during remission (T-698) has the same restriction fragments as the donor cell DNA. DNA isolated during relapse (T-741) has the same restriction fragments as the donor cell DNA, but there is much more of the 11-kb than the 12-kb restriction fragment. Admixtures of 5% to 50% recipient DNA and 95% to 50% donor DNA are shown at right.](image-url)
obtained during the November 19, 1986 remission (698-T) and donor-specific (D) and recipient-specific (R) restriction fragments. 95% to 50% donor DNA are shown at right. During relapse, the DNA has both donor- and recipient-specific RFLPs but recipient-specific only donor-specific RFLPs are seen. During remission, the DNA has donor-specific RFLPs predominate. Admixtures of 5% to 50% recipient DNA and 45% to 50% donor DNA are shown at right.

Thus, the simplest interpretation is that the malignant clone arose from a recipient cell post-BMT leukemic relapse. Since the January bone marrow sample was primarily comprised of recipient cells, these cells were completely invisible using cytogenetic techniques. It has been suggested that different subpopulations of cells may be responsible for the predominant karyotype observed depending on whether cytogenetic analysis is performed directly or following short-term culture. In this case, however, both direct and short-term culture methods failed to uncover recipient cells. In future studies, the use of cell-sorted marrow populations might also help produce a sample enriched for leukemic cells for more definitive cytogenetic and molecular analyses.

Two explanations for the apparent absence of recipient cells in the cytogenetic spreads are possible. First, most of the polyploid cells observed could not be karyotyped. It is possible that these cells were very aberrant leukemic recipient cells. If this were the case, it is surprising that a diploid precursor cell of recipient lineage was never detected. For any given bone marrow sample, 11% mosaicism could be excluded with 90% to 99% confidence. For all of the samples cumulatively analyzed, 2% mosaicism could be excluded with 99% confidence. A second possibility is that even the polyploid cells that contained four X chromosomes were derived from a recipient cell that lost the Y chromosome and gained an X chromosome by abnormal segregation and then underwent a non-disjunctive mitosis that resulted in tetraploidy. A similar mechanism was proposed by Stamberg et al\textsuperscript{2} to explain a case of ALL in which there was a shift from heterozygosity to homozygosity for a large chromosome polymorphism on chromosome 15.

In light of the results reported here, it is interesting to review the basis on which donor cell relapse in BMT patients has previously been established.\textsuperscript{3} Of the 14 cases that have been reported to date, only three use molecular data and in each of these cases, only one DNA probe was used on one bone marrow sample.\textsuperscript{4,5,7,9} Two cases of donor relapse were identified based on expression of isoenzymes.\textsuperscript{12,14} Nine cases were identified as donor relapse based strictly on cytogenetic data. The cytogenetic evidence supporting the cases described by Elfenbein et al\textsuperscript{2} and by Smith et al\textsuperscript{11} is the most comparable to the cytogenetic evidence presented in this report. In both of these cases a male patient was transplanted with female bone marrow cells that also show one chromosomal polymorphism. On relapse, no Y chromosome is detected in the bone marrow cells and 45, XO cells contain the donor chromosome polymorphism. The conclusions of Newburger et al\textsuperscript{a} are based strictly on a polymorphism that distinguished donor and recipient chromosome 21. In several cases of post-BMT relapse, cytogenetic abnormalities associated with the leukemic state were observed in donor cells.\textsuperscript{4,8,10,11} This evidence suggests that donor cells are involved in the relapse though it does not preclude the possibility of recipient cell involvement.

The present case demonstrates that caution must be exercised when assigning the leukemic cell lineage in cases of post-BMT relapse. Cytogenetic analyses alone may not always detect the transformed cell line. Interpretation of molecular analyses may be difficult if the DNA probes used do not detect both donor-specific and recipient-specific
restriction fragments. In addition, molecular analyses may be confounded by the loss of specific chromosomes, or by the reemergence of non-leukemic cells of recipient origin. Thus, it is essential to use DNA probes that are informative for loci on more than one chromosome and to establish that the relapse is associated with a shift in the RFLPs of the transplanted bone marrow from a donor to a recipient pattern. An accurate assessment of the frequency and possible mechanisms of donor cell relapse will require long-term follow-up of post-BMT patients using both cytogenetic and molecular analyses of bone marrow and peripheral blood.

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