Molecular Genetic Rearrangements Distinguish Pre- and Post-Bone Marrow Transplantation Lymphoproliferative Processes

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Chronic myelocytic leukemia (CML) may display a lymphoproliferative phase (lymphoid blast crisis) that is generally of B cell phenotype. Since lymphoproliferative disorders may occur following bone marrow transplantation (BMT), it may be difficult to distinguish posttransplant relapse of CML lymphoid blast crisis from de novo lymphoproliferation. Lymphoid blast crisis cells from a patient with CML displayed immunoglobulin heavy chain gene (Cμ) rearrangement before BMT. Following BMT the patient developed a lymphoproliferative disorder involving multiple organs. Clonal rearrangement of Cμ was demonstrated in several involved tissues. The rearranged Cμ restriction fragment was distinct from that displayed before BMT. Additionally, rearrangement of the breakpoint cluster region (bcr) was demonstrated in the pretransplant blast crisis sample, but not in the posttransplant lymphoproliferation samples, thus confirming that these lymphoproliferative disorders were distinct. Molecular genetic techniques offer powerful diagnostic tools for monitoring the course of patients with CML undergoing BMT.

THE 9;22 CHROMOSOMAL translocation, which results in the Philadelphia chromosome typical of chronic myelocytic leukemia (CML), causes alterations (or rearrangements) of the breakpoint cluster region (bcr) located on the long arm of chromosome 22. In CML patients with the Philadelphia chromosome such rearrangements of the bcr can be demonstrated by the use of Southern blotting and genomic probes for this region.1,2,3 CML frequently terminates in lymphoid blast crisis that is generally of B cell phenotype with expression of B lineage surface antigens and often with cytoplasmic immunoglobulin.4 In many cases of lymphoid blast crisis, immunoglobulin heavy chain rearrangement and in some cases light chain rearrangement can be demonstrated,5,6 indicating a monoclonal cell population. Following bone marrow transplantation (BMT), lymphoproliferative disorders may develop7,8 that can be difficult to distinguish from relapse of the primary disease, eg, CML. We report the use of immunoglobulin and bcr probes to distinguish posttransplant lymphoproliferation from relapse of lymphoid blast crisis in a patient with CML.

MATERIALS AND METHODS

Patient materials. The patient was a 12-year-old boy (UPN 588) with an established diagnosis of Philadelphia chromosome positive CML. He presented for transplant evaluation following lymphoid blast crisis. Induction chemotherapy returned his marrow morphology to a chronic phase appearance, and he underwent further chemotherapy plus transplantation using haploidentical bone marrow from his mother; the bone marrow was depleted of mature T lymphocytes. Although he showed early engraftment, he became acutely ill with the abrupt onset of lymphadenopathy and fever on post-BMT day 30. Cervical node biopsy confirmed a diagnosis of lymphoproliferative process. In spite of attempted therapy he died 43 days after BMT. Pleural fluid, lung, lymph node, CSF, and bone marrow all showed involvement with lymphoblastoid cells. Procedures and studies were performed in accordance with institutional guidelines and with informed consent.

An enriched mononuclear cell population from peripheral blood during pre-BMT blast crisis was obtained by Ficoll-Hypaque centrifugation (45 minutes, 400 X G, room temperature). Cells were immediately frozen in RPMI with 10% dimethylsulfoxide and stored at −70°C. Immunophenotyping was done on fresh cells. Tissue specimens obtained at thoracotomy and at autopsy were frozen in liquid nitrogen.

Fresh peripheral blood cells and frozen tissues were studied for immunophenotype and extracted DNA was studied for gene rearrangements (as outlined below) for all samples.

Immunophenotyping. Cells were assessed for the presence of surface antigens associated with myeloid cells and with B and T lineage lymphocytes. For these studies cells were mixed with monoclonal antibody and incubated for 30 minutes at 4°C, washed and incubated with fluorescein-labeled anti-mouse antibody for an additional 30 minutes at 4°C. After three washes, cells were analyzed by Fluorescence Activated Flow Cytometry (Orthospectrum 3, Ortho Diagnostics Systems, Raritan, NJ, or FACS 4, Becton-Dickinson, Mountain View, CA). Appropriate controls using no monoclonal antibody were included to assess autofluorescence and control mouse ascites was used to determine background staining. Fluorescence profile was generated for each antibody tested, allowing percentage of antigen-positive cells to be determined. If more than 20% of cells tested were positive for a given antigen, these cells were considered to be positive for that antigen.

Monoclonal antibodies used in these studies included 35.1 (CD2), TA-1 (CD11a), which recognize T-cell associated surface antigens. Antibodies used to assess B lineage associated surface antigens included BA-1 (CD24), BA-2 (CD9) (found both on B cells and on granulocytes), and BA-3 (common acute lymphoblastic leukemia antigen, CD10). B1 (CD20) and B4 (CD19) were also used for some samples. MCS-2 recognizes a 150 kd glycoprotein (CDw13) present on myeloid and monocytic cells. This antibody does not bind to lymphoid cells of either B or T lineage. The OKM1 antibody was used to detect CD11 expression. Antibodies to HLA-DR and common leukocyte antigen (CLA) were also used. In addition, cells from the posttransplant specimens were stained with antibodies.
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chains. Bakhshi et al.9 Briefly, cells were suspended in 5 mL of cold Forencse buffer, then 15 mL cold Forencse-Triton X-100 buffer was added followed by gentle mixing to resuspend cells. Cells were pelleted (1,500 rpm for ten minutes at 4°C), then resuspended in 25 mL Forencse-Triton buffer. Following pelleting, cells were washed twice in Forencse and then resuspended in 4.25 mL Forencse buffer, 1 mL 0.5 mol/L EDTA, proteinase K (2 mg/5 mL total solution). Sodium dodecyl sulfate (SDS) (10%) was added to final concentration of 1% (0.45 mL) and this mixture incubated at 37°C for three hours, following which 5 mol/L NaCl was added to final concentration of 0.5 mol/L NaCl. An equal volume of 1:1 mix of phenol and chloroform isoamyl alcohol (24:1) (CIAA) were added, mixed, and centrifuged (2,000 rpm for five minutes). The aqueous (top) layer was recovered and re-extracted, first using phenol and CIAA and then using CIAA. Cold (−20°C) absolute ethanol (2.5 vol) was added to the aqueous DNA solution. Following overnight incubation (−20°C) the DNA pellet was centrifuged (10,000 to 12,000 rpm for fifteen minutes at 4°C), ethanol discarded, the pellet dried, and resuspended in TE at a DNA concentration of approximately 1,000 µg/mL.

Solid tissues were first minced and homogenized in 5 mL TNE (.01 mol/L Tris, .01 mol/L NaCl, .001 mol/L EDTA) buffer. Proteinase K (100 µg/mL final concentration) and 250 µl 10% SDS and 250 µl β-mercaptoethanol were added and the mixture incubated for three hours, 50°C. The mixture was then extracted three times (1 vol phenol, then 1 vol of phenol:CIAA mix, then 1 vol of CIAA). Sodium acetate (1/6 vol of 3M) was added to the aqueous DNA mixture before addition of 2.5 vol of cold absolute ethanol. The DNA was pelleted, dried and resuspended as outlined above.

Detection of gene rearrangement. Gene rearrangement studies used the gel transfer technique described by Southern.10 High molecular weight DNA was digested to completion using BamHI restriction endonuclease. Restriction fragments were size separated by electrophoresis through a 0.7% agarose gel at 4 V for 36 hours (Cγ) or 0.8% agarose for 24 hours (bcr). Size separated DNA fragments were then transferred from the gel to nylon filter (Zetabind, AMF Cuno, Menedin, CT) in 20 x SSC. Following washing and prehybridization (with salmon sperm DNA) filters were hybridized in the presence of 50% formamide at 42°C for 24 hours using DNA probes labeled to high specific activity with 32P-cytosine using random primer method.11 Filters were washed for fifteen minutes in 2 x SSC, 0.1% SDS followed by a single wash with 0.1 x SSC, 0.1% SDS at 60°C. Filters were then exposed to x-ray film (Kodak, Rochester, NY) for autoradiography for twelve to 24 hours at −70°C.

Probes. Immunoglobulin gene heavy chain rearrangements were detected using a 1.3 kb DNA genomic probe for the heavy chain constant region (Cγ) and κ-light chain rearrangements were studied with a 2.5 kb genomic constant region probe (Cκ), both provided by S.J. Korsmeyer. Human bcr (PR-1) probe obtained from Oncogene (Mineola, NY) is a 1.2 kb HindIII-BglII restriction fragment of genomic DNA originally cloned from the breakpoint region of a patient with CML1 and corresponds to an area in the intron between the third and fourth exons of the breakpoint cluster region.2 In the formation of the Philadelphia chromosome, this portion of the bcr is usually translocated to chromosome 9.

Restriction fragment length polymorphisms (RFLP). RFLP were analyzed before and following BMT by Southern blotting as previously described.12 For these studies, DNA was digested with TaqI and hybridized with probe PDP 34 (a gift from Dr David Page). This test is routinely performed by the Molecular Diagnostics Laboratory for pre- and post-BMT samples.

RESULTS

Samples obtained before BMT at the time of lymphoid blast crisis and from the posttransplant episode showed lymphoblastic morphology. Cell marker studies showed that lymphoid cells obtained before transplant were HLA-DR, CD10 and CD9 positive, but were negative for CD2. These data were consistent with lymphoid blast crisis involving B-cell precursors. Posttransplant cells, however, were negative for all B cell, T cell, and most myeloid markers tested, although both HLA-DR and CLA were displayed. The posttransplant sample also did not display CD20 or CD19. Stains for cytoplasmic immunoglobulin showed cytoplasmic γ, α, and μ heavy chains in addition to κ and λ light chains, indicating a small polyclonal cell population in the lung tissue. The majority of cells from the lung tissue and all cells

![Southern blot analysis of immunoglobulin genes in pre- and post-BMT samples.](https://example.com/southern-blot.png)

**Fig 1.** Southern blot analysis of immunoglobulin genes in pre- and post-BMT samples. Genomic DNA was digested with BamHI and probed with a Cγ immunoglobulin heavy chain constant region probe (left arrow). Posttransplant samples that histologically represented lymphoma involving lymph node (LN), pleural fluid (PF), and lung samples all showed a 5.7 kb rearranged Cγ fragment (right arrow). The germline DNA band position (lane Germ – placental DNA) is marked by a solid line on each side.
from the perihilar lymph node failed to stain for either cytoplasmic or surface immunoglobulin.

Molecular genetic studies confirmed the impression that the pre-transplant blast crisis cells were of B cell lineage, showing a clonal proliferation containing a 5.4 kb BamHI Cμ-hybridizing fragment (Fig 1). The posttransplant samples also showed rearrangement of the Cμ region, resulting in a 5.7 kb BamHI Cμ restriction fragment (Fig 1) that was identical for the three samples studied (lymph node, pleural fluid, and lung), but which was different from the pretransplant blast crisis-associated rearrangement. The κ-light chain (Cκ) was in the germline configuration in pre- and post-BMT samples. Analysis of the post-BMT lymphoproliferation for RFLP showed that these samples were entirely of donor cell origin. In studies of bcr, BamHI-digested DNA from blast crisis showed rearrangement of bcr, whereas tissue from the three posttransplant samples show only germline bcr configuration (Fig 2).

**DISCUSSION**

This patient presented a somewhat difficult diagnostic challenge in that he developed a lymphoid malignancy during the very early post-BMT course. Cell marker studies were inconclusive because of the heterogeneity in the various tissues studied. No B cell associated surface antigen was detected, although cytoplasmic γ, α, and μ heavy chains were detected as were κ and λ light chains. The nonstaining population of cells might represent a monoclonal population, but cell marker studies could not definitively demonstrate monoclonality. Immunoglobulin gene rearrangement has been demonstrated in both lymphoid blast crisis6 and in Epstein-Barr virus associated posttransplant lymphoproliferative disorders.7 The direct comparison of immunoglobulin gene rearrangements allowed us to say that this posttransplant lymphoid proliferation represented a different monoclonal disorder than that resulting in lymphoid blast crisis in this patient. In addition, these studies showed that the various sites of posttransplant lymphoproliferation involvement represented cells derived from a single clone as opposed to a multiclonal disorder arising simultaneously in different sites, since the Cμ rearrangement was identical in all three samples. These data strongly suggest that this posttransplant lymphoproliferation represented a new B cell disorder unrelated to the pre-BMT lymphoid blast crisis. The presence of bcr rearrangement before but not after BMT supports this interpretation.

The post-BMT samples show deletion of one and rearrangement of one Cμ allele, providing evidence of a monoclonal population of cells. The lack of germline band suggests that some polyclonal B-cells detected by markers may be dually rearranged for Cμ and that cells retaining one germline allele may be present in numbers too small to detect. The large population of cells that represents the monoclonal lymphoproliferative disorder does not show cytoplasmic μ, suggesting that the detected Ig heavy chain rearrangement is nonproductive. The presence of germline kappa gene is also consistent with a non-productive Ig heavy chain rearrangement.

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**Table 1. Cell Marker Studies**

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Abbreviation: NT, not tested.

* A polyclonal population staining for cytoplasmic Ig represents a small number of cells; a large percentage of cells show no staining for either surface or cytoplasmic immunoglobulin.

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**Fig 2.** Southern blot analysis of bcr gene in pre- and post-BMT samples. Genomic DNA was digested with BamHI and probed with a genomic probe for the bcr region of chromosome 22. The pre-BMT peripheral blood sample shows a rearranged fragment (arrow), while the post-BMT samples show only germline configuration of bcr. Germline band size is denoted by solid lines.
Molecular genetic techniques offer powerful tools for diagnosing and monitoring the course of a variety of hematological malignancies. Probing of the breakpoint cluster region in CML has now been demonstrated to be an effective means of following the course of CML patients post-transplant. A previous report by Bartram et al. showed that this method may predict CML relapse following BMT before the detection of the Philadelphia chromosome by standard cytogenetics. We now have described a case in which possible post-BMT relapse was ruled out by the presence of bcr rearrangement before but not following bone marrow transplantation and on the basis of unique immunoglobulin gene rearrangements. As more effective and specific therapies become available, it will be extremely important to distinguish between new malignancies and those which represent posttransplant relapses. Such studies are of value in interpreting the results of BMT.

REFERENCES

Molecular genetic rearrangements distinguish pre- and post-bone marrow transplantation lymphoproliferative processes

WJ Miller, RS Shapiro, R Gonzalez-Sarmiento and JH Kersey