A Monoclonal Immunoblastic Sarcoma in Donor Cells Bearing Epstein-Barr Virus Genomes Following Allogeneic Marrow Grafting for Acute Lymphoblastic Leukemia

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A patient undergoing marrow grafting for acute lymphoblastic leukemia from his partially HLA-mismatched sister displayed a widely disseminated immunoblastic sarcoma at autopsy. The tumor was monoclonal by immunoglobulin light-chain staining. Blot hybridization analysis, using a cloned highly polymorphic locus in human DNA as a probe, showed the tumor to be of donor-cell origin. Cytogenetic analysis also demonstrated donor-cell origin. Blot hybridization analysis demonstrated Epstein-Barr virus (EBV) genomes in the tumor. By contrast, reexamination of material from a previously reported case of a donor-type relapse showed no evidence of EBV DNA. In neither case was there evidence of cytomegalovirus DNA. This study documents the association of EBV with a malignant, monoclonal B-cell lymphoma arising in a marrow graft recipient. We conclude that DNA restriction fragment length polymorphisms can be used to prove the origin (donor or host) of neoplastic relapse following allogeneic marrow grafting. Further, cell types different from those of the original leukemia may be involved.

A LTHOUGH LEUKEMIC RELAPSE in patients undergoing marrow grafting often results from failure to eradicate the original malignant cell population, we and others6,7 have observed neoplastic transformation of donor cells. In all cases studied to date, the cell of origin (donor or host) has been established using karyotyping, Y-chromatin staining, or other cytogenetic markers, or white blood cell isozyme polymorphisms. Cytogenetic analysis requires either sex mismatched donor–recipient pairs or the existence of a discriminating cytogenetic marker. An alternative approach to this problem exploits fragment length polymorphisms in regions of the human genome recognized by restriction endonucleases. For example, the identification of a highly polymorphic genetic locus in the human genome makes it possible to distinguish individual genotypes. We have used this technique to study an immunoblastic sarcoma that followed allogeneic marrow grafting for acute lymphoblastic leukemia. The tumor consisted of a monoclonal proliferation of immunoglobulin-containing cells containing Epstein-Barr virus DNA.

CASE REPORT

A 5-yr-old black male (unique patient number 1085) developed fever, anemia, and leukopenia in December 1977. A bone marrow examination suggested the diagnosis of acute lymphoblastic leukemia; however, his blood counts and marrow morphology improved spontaneously. In February 1978 he developed anemia and leukopenia, and the bone marrow was packed with periodic acid Schiff (PAS)-positive lymphoblasts. A remission was induced with vincristine, prednisone, and L-asparaginase. He received intrathecal methotrexate and cranial irradiation and was maintained on 6-mercaptopurine. He was in remission when he suffered biopsy-proven Pneumocystis carinii pneumonia in April 1978, which was successfully treated with pentamidine. In August 1979 he relapsed, and a second remission was induced with vincristine, prednisone, and L-asparaginase, which was maintained on vincristine, methotrexate, and L-asparaginase. He was referred for marrow grafting from his 6-mo-old sister.

On admission, the patient had a palpable spleen and normal blood counts. The marrow showed clusters of lymphoblasts consistent with relapse, and the cerebral spinal fluid (CSF) contained leukemic cells. The patient was HLA type A2,34, B18, B35 and the donor A2,3, B18, B35, the A locus mismatch being due to a paternal chromosome recombination. Their cells were not reactive in mixed leukocyte culture. The patient’s red cell type was A positive and the donor AB positive.

The patient was prepared with 60 mg/kg cyclophosphamide on days –4 and –3, intrathecal methotrexate on days –6 and –1, plasma exchange with 3.5 liters AB plasma and 500 ml of B-positive red cells on day –1, and 1000 rad of total body irradiation on day zero. He received 2.5 x 10⁹ nucleated marrow cells from his sister. He received granulocyte transfusions from his mother on days 6 through 17 and 12 mg of methotrexate IV on day 1 and 8 mg on days 3, 6, 11, 18; 12 mg intrathecally on days 32 and 46.

Bone marrow examination showed engraftment on day 14. Blood cultures on day 8 grew protonobacter and peptococcus and he was treated with ticarcillin and gentamicin through day 32. Ampicillin B (0.3 mg/kg) was given on days 33 through 53 because of persistent fever and Candida colonization. He developed biopsy-proven graft-versus-host disease (GVHD) of the skin by day 25 and was treated with prednisone 2 mg/kg. Subsequently, horse antilymocyt globulin (15 mg/kg) was added and continued from day 32 until death.10 Liver function test abnormalities consistent with GVHD began on day 35 with a bilirubin of 3, alkaline phosphatase of 100–150, and SGOT of 40–50. By day 49 the bilirubin was 24, SGOT 85, and alkaline phosphatase 161. An abdominal ultrasound...
showed hepatosplenomegaly with a question of "sludge" in the gall bladder. On day 49 he developed abdominal pain and distension. On day 54 he developed increased abdominal pain and metabolic acidosis. An abdominal x-ray showed distended loops of bowel. Because of the possibility of finding a surgically remediable lesion, a laparotomy was performed that showed acalculous cholecystitis and hepatosplenomegaly. Cardiac arrest occurred during surgery and attempts at resuscitation failed.

MATERIALS AND METHODS

Autopsy material:
A complete autopsy was performed 2.5 hr after death. Tissue from all organs, including the central nervous system, was fixed in Millonig's bufferd formalin and embedded in paraffin. Sections of multiple organs, including lungs, liver, spleen, lymph nodes, adrenals, and kidneys, were frozen at -70°C in Optimal Cutting Temperature Compound (OCT. Miles Laboratories, Inc., Elkhart, Indiana). Sections of liver less than 1 mm thick were placed in Karnovsky's EM fixative, subsequently postfixed in osmium and S. Collidine, dehydrated, and embedded in Epon 812. Immediate bacterial, fungal, and viral cultures of both lungs, liver, spleen, and the right kidney were obtained.

Immunoperoxidase Staining

Sections of lymph node and liver that had been fixed in formalin and embedded in paraffin were stained for intracytoplasmic immunoglobulin (clg) and muramidase, using the peroxidase-antiperoxidase (PAP) technique.12,13 The specificity of the rabbit antimmunoglobulin sera (Dako Corporation, Santa Barbara, Calif.) was confirmed with human myeloma tissue characterized previously. Optimum serum dilutions were determined by staining human tonsil and ranged from 1:1000 for anti-alpha heavy chains to 1:3200 for anti-lambda light chains.

Detection of DNA Polymorphisms

Pretransplant skin punch biopsy specimens were frozen in liquid nitrogen, thawed at the time of relapse, and grown in Waymouths MB 752/1 medium. Cells were harvested from 80 tissue culture bottles and high molecular weight DNA isolated as described elsewhere.14 Autopsy tissue was frozen at -20°C and DNA extracted by the same method. Ten micrograms of DNA were digested with EcoRI (New England Biolabs), electrophoresed in 0.5% agarose, and transferred to nitrocellulose by the method of Southern.15 Plasmid pAW101 in the E. coli host HB101 was obtained from R. White1 and purified by the acid phenol method16 following growth in L broth with 50 µg/ml tetracycline and enhancement with 200 µg/ml chloramphenicol. The cloned insert was purified from the plasmid by EcoRI digestion by agarose gel electrophoresis and electroelution,17 and labeled by nick translation18 to a specific activity of 2 x 106 cpm/µg. Hybridization of the labeled probe with DNA on the nitrocellulose filters was done under the conditions described by Wahl et al.19 After hybridization, blots were washed as previously described14 and autoradiograms prepared using Kodak XAR film.

Detection of EBV DNA

Southern blots of tumor cell DNA digested with EcoRI were hybridized with intact EB virion DNA or with plasmids containing the Bam HI W or EcoRI B fragments of EBV (FF41) DNA.20,21 The number of EBV DNA copies per cell was estimated by nucleic acid spot hybridization.22 The standard was intact viral DNA and the probe consisted of 32P-labeled plasmids containing Bam HI W and EcoRI B and C fragments (approximately 70% of the genome).

Cytogenetics

Peripheral blood lymphocytes from the patient taken prior to conditioning for grafting and from the donor were prepared as for routine chromosome investigation. Also, bone marrow from the patient taken prior to conditioning was prepared by the routine direct method for cytogenetic analysis, but marrow from the donor was not available. Autopsy specimens of a lymph node were teased apart and single cell suspensions were prepared. The suspensions were washed 3 times in sterile phosphate-buffered saline and cultured in Waymouths MB 752/1 with 15% fetal calf serum and 0.1% antibiotic-antimycotic for 18–36 hr. The final 1.5 hr of incubation was carried out in the presence of 0.03 µg/ml of colcemid, and the cells were then harvested and slides were prepared. The slides were stained with quinacrine and 100 large mononuclear cells were scored for the presence of Y-chromatin.23 Metaphase spreads were photographed for analysis. Peripheral blood and bone marrow slides of the patient prior to conditioning and peripheral blood slides of the donor were also stained with quinacrine, and mononuclear cells were scored for Y-chromatin.

RESULTS

Autopsy Findings

Microscopically, there was a widespread prominent lymphoid proliferation best characterized as an immunoblastic sarcoma of B cells.11 It was diffuse, with no suggestion of nodularity (Fig. 1). In sections of 15 lymph nodes from the cervical, thoracic, and abdominal areas, the normal architecture was replaced predominantly by immunoblasts with a moderate amount of basophilic and strongly pyroninophilic cytoplasm.

Fig. 1. Massive neoplastic infiltration of kidney interstitium adjacent to a glomerulus (2-µm methacrylate section, Jones' silver methenamine X128). The large immunoblasts have a prominent central nucleus. Cells with more abundant cytoplasm and smaller eccentric nuclei resemble plasma cells (small arrows). A mitotic figure is present (large arrow).
They had atypical nuclei and 1–3 prominent nucleoli. In addition, smaller cells with even more striking plasmacytoid features were present. This lymphoid proliferation heavily involved the splenic red pulp and peripheral areas, peribronchial and perivascular portions of the pulmonary interstitium, the hepatic portal zones, the kidney interstitium, and the predominantly perivascular areas of the bone marrow stroma. The overall marrow cellularity was approximately 50% of normal and consisted of equal proportions of the malignant infiltrate and morphologically normal cells of the three hematopoietic lines (Fig. 2). Tumor focally involved other sites, including the mucosa and submucosa of the stomach and small and large intestine; the subepithelium of the ocular conjunctiva, esophagus, gallbladder, and urinary bladder; and the interstitium of lacrimal and salivary glands as well as prostate. The spinal cord, brain, testes, myocardium, or other cardiac structures were uninvolved.

Electron micrographs of the tumor infiltrate in the liver demonstrated poor preservation of subcellular organelles. There was no evidence of viral structures. All viral, fungal, bacterial cultures were negative, aside from the growth of 5 colonies of coagulase-negative staphylococci from the left lung.

**Immunoglobulin Studies**

There was strong cytoplasmic staining of a majority of tumor cells for lambda light chains, often concentrated in a paranuclear location (Fig. 3). This was positive at a maximum antibody dilution of 1:6400. Tumor cell staining for kappa, gamma, alpha, and mu chains was negative. Scattered histiocytes and plasma cells that stained with multiple antisera comprised less than 3% of the lymphoid population in areas of tumor infiltration. There was no monoclonal serum protein component.

**Cytogenetic Analysis**

Cytogenetic analysis of the patient’s cells before grafting showed 46 XY with 99% Y-chromatin positive (Table 1). The donor’s metaphases were 46 XX. The donor’s cells were 18% Y-chromatin positive and quinacrine staining showed a bright fluorescent centromere on one chromosome no. 3. After grafting, 28 metaphases from the patient’s marrow all showed 46 XX chromosomes and 10% of the cells were Y-chromatin positive. Cytogenetic analysis of cultured cells derived from tumor-bearing portions of lymph nodes showed 16% Y-chromatin positive mononuclear cells, and 50 metaphases all demonstrated a normal female karyotype without evidence of translocation using the Q-banding technique.

**Blot Hybridization Analysis of Tumor**

High molecular weight DNA was isolated from a liver tumor mass and from cultured skin fibroblasts from both donor and recipient. The DNA was analyzed by restriction endonuclease digestion with EcoRI and Southern blot hybridization as described above. Figure 4 shows the result of this analysis. The pAW101 probe detected an EcoRI fragment of about 16,000 base pairs in DNA from the recipient’s normal skin fibroblasts, while DNA extracted from both the tumor and the donor’s normal skin fibroblasts contained two such bands at around 16,000 and 15,000 base pairs. We concluded that the tumor cells contained donor DNA, and the tumor was therefore of donor origin.

Because transformation by potentially oncogenic viruses could provide an explanation for donor-cell neoplastic transformation, we assayed for the presence of Epstein-Barr virus and cytomegalovirus genomes. Tumor DNA digested with EcoRI and blotted on nitrocellulose filters was found to contain Epstein-Barr virus DNA (Fig. 5). Controls comparing the restriction endonuclease digestion patterns with normal viral DNA and cloned fragments of the viral genome showed that at least 70% of the EBV genome was present. Quantitation of the amount of EBV-specific DNA showed that there were about seven genome equivalents per cell. The donor was seropositive for EB viral capsid antigens and EB nuclear antigen. No EBV antigens were found in thawed samples of previously

**Fig. 2.** Large immunoblasts with irregular nuclear margins in touch imprint of bone marrow from autopsy (Wright-Giemsa stain, X320). A normoblast is present at upper left.
Fig. 3. Monoclonal lambda light chain staining of neoplastic cells in hepatic portal areas (horseradish peroxidase–antiperoxidase with diaminobenzidine, hematoxylin counterstain X320). (A) The absence of immunoblast staining with anti-kappa serum. A cell resembling a histiocyte (arrow) is positive. (B) A majority of the immunoblasts show focal cytoplasmic staining (arrows) with anti-lambda serum.
Table 1. Summary of Cytogenetic Studies Prior to and Following Transplantation for Patient No. 1085

<table>
<thead>
<tr>
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<th>Metaphase Karyotype (Number Examined in Parentheses)</th>
<th>Y-Chromatin Positive in Interphase Nuclei</th>
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<tr>
<td>Pregraft</td>
<td></td>
<td></td>
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<tr>
<td>Patient — marrow</td>
<td>(20) 46,XY</td>
<td>93%</td>
</tr>
<tr>
<td>PB-PHA*</td>
<td>(20) 46,XX</td>
<td>18%</td>
</tr>
<tr>
<td>Donor PB-PHA</td>
<td>(28) 46,XX</td>
<td>10%</td>
</tr>
<tr>
<td>Postgraft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient — marrow</td>
<td>(50) 46,XX</td>
<td>16%</td>
</tr>
<tr>
<td>PB-PHA</td>
<td>(15) 46,XX</td>
<td></td>
</tr>
<tr>
<td>Tumor — lymph node</td>
<td>(50) 46,XX</td>
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*PB-PHA, phytohemaglutin-stimulated peripheral blood cells.

frozen tumor tissue, and cocultivation studies of the same frozen tumor failed to give evidence of EBV. The same blot analyzed with cloned fragments of the CMV genome was negative. Because of these findings, we restudied material from a previously reported donor-type relapse in a patient with ALL (unique patient number 49). Table 2 compares a summary of the data from the two cases. Extensive pretransplant phenotypic characterization of this case had not been done; however, both the original and recurrent leukemia were morphologically similar and consistent with ALL. Also, in that case, normal recipient material was unavailable for DNA polymorphism analysis. No evidence of either EBV or CMV was found in the previous case.

DISCUSSION

The cases of donor relapse reported here and elsewhere illustrate several significant points. The first is that DNA polymorphisms recognized by restriction endonuclease digestion and Southern blot hybridization analysis can be used to determine the donor or host origin of neoplastic disease appearing following allogeneic marrow transplantation. In this case we have exploited the existence of a cloned, highly polymorphic locus in human DNA to demonstrate the occurrence of a neoplasm in donor cells. Previous documentation of donor-type relapses has relied on cytogenetic analysis or white blood cell isoenzyme polymorphisms. Karyotyping sex chromosomes and Y-chromatin staining are limited to cases of opposite sex donor–recipient pairs. Cytogenetic abnormalities can be used as reliable markers, but unambiguous chromosome banding patterns are often not readily identifiable in clinical tumor samples. White cell isoenzyme polymorphisms can be obscured by contaminating red cells. This new technique avoids these technical problems and is readily applicable to any case in which material containing a preponderance of tumor cells can be obtained and from which relatively small amounts (20–50 µg) of DNA can be isolated. In addition, restriction fragment length polymorphisms are known to exist in the human beta-globin and tubulin gene regions. The combined use of these markers should make it possible to distinguish almost any two individuals except identical twins.

A second noteworthy point is that donor neoplasms can be of a different cell type than the original host leukemia. The recurrent tumor reported was a monoclonal immunoblastic sarcoma of B cells that contained only lambda light chains. Gosset et al. have reported a case of immunoblastic sarcoma following transplantation for AML, in which tumor cells contained cytoplasmic IgM (lambda) and there was a serum IgM (lambda) M-component. Interestingly, both cases involved donor–recipient pairs bearing an HLA mismatch. The present case had one A-locus mismatch, while the donor and recipient in the previous case were reactive in MLC. Also, both cases developed severe, biopsy-proven skin GVHD. Other cases of donor cell neoplasms have been characterized, either morphologically or in more precise detail, as recurrent leukemias of the same type as the original leukemia.

Fig. 4. Autoradiographs of EcoRI-digested DNA. The filter was hybridized with 32P-labeled human DNA excised from the pAW101 clone. DNA from the recipient’s fibroblasts (lane A) contain one band of 16 kb, while DNA from the recurrent tumor (lane B) and the donor’s fibroblasts (lane C) contain bands of 15 and 16 kb. The locations of molecular weight markers, in kilobase pairs, are included on the right.
The third point is that we have detected EBV DNA in relatively low copy number in tumor tissue by the restriction endonuclease blot hybridization technique. EBV has been reported in association with abnormal lymphoid proliferation among immunosuppressed renal transplant recipients and recipients of thymic epithelium transplants, in patients with immunologically related diseases such as rheumatoid arthritis and systemic lupus erythematosus, and in patients with heritable immunodeficient states such as ataxia telangiectasia or the X-linked lymphoproliferative syndrome. Many of these proliferations have been classified histologically as immunoblastic or reticulum cell sarcomas. When clonality has been investigated by immunoglobulin staining, the tumors have been polyclonal. We were unable to detect EBV antigens (viral capsid antigen, early antigens, and nuclear-associated antigens) in the present case. Gossett et al. also did not detect EBV antigens. This could be due to nonexpression of viral genes or inadequate preservation of previously frozen tissues. The use of the hybridization techniques avoids this ambiguity.

Table 2. Recurrence of Malignancy in Donor Cells Following Marrow Allograft

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>HLA histocompatibility</th>
<th>Conditioning therapy</th>
<th>Clinical acute GVHD</th>
<th>Postransplant immunosuppressive therapy</th>
<th>Recurrent malignancy</th>
<th>Histology</th>
<th>Markers</th>
<th>Cytogenetics</th>
<th>WBC isoenzymes</th>
<th>DNA polymorphisms</th>
<th>Blot hybridization analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN 1085</td>
<td>Male, 5 yr</td>
<td>Matched</td>
<td>Cyclophosphamide and TBI 1000 RAD</td>
<td>Grade III</td>
<td>Methotrexate, prednisolone, horse antithymocyte globulin</td>
<td>56 Days</td>
<td>Immunoblastic sarcoma</td>
<td>clg: lambda light chain positive; kappa light chain and mu, alpha, and gamma heavy chain negative, muramidase, and chloroacetate esterase negative</td>
<td>90% normal female diploid; 10% Y chromatin positive</td>
<td>Inconclusive</td>
<td>Donor cell malignancy</td>
<td>CMV negative</td>
</tr>
<tr>
<td></td>
<td>Sister, 6 mo</td>
<td>A locus mismatch</td>
<td>Grade III</td>
<td>ND</td>
<td>Inconclusive</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>UPN 49*</td>
<td>Female, 16 yr</td>
<td>Matched</td>
<td>TBI 1000 RAD</td>
<td>Grade I</td>
<td>Methotrexate</td>
<td>62 Days</td>
<td>Acute lymphoblastic leukemia</td>
<td>Cell surface: Ig, E, EA, EAC, and Ia negative, 10.2† positive</td>
<td>100% normal male diploid</td>
<td>ND</td>
<td>CMV negative</td>
<td>EBV negative</td>
</tr>
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</table>

*From reference 1.
† 10.2 is a monoclonal antibody against a T-cell differentiation antigen also found on malignant B-cell lines. ND, not done.
The data collected in this case cannot distinguish whether the EBV genomes detected in the tumor cells represent an infection unrelated to etiology, or whether they are a direct or indirect cause of the neoplastic change. The tumor cells in this case lacked the (8;14) cytogenetic abnormality described in some candidate EBV-induced neoplasms. The marrow donor was infected with EBV by serologic criteria and may well have been the source of the virus detected in the donor tumor cells. The recipient was also seropositive, but it was not possible to determine whether this was a response to viral antigens or represented passive transfer of antibody from blood products. Even if EBV is implicated in some of the donor cell neoplasms following allogeneic marrow engraftment, it cannot be the entire explanation, as indicated by the absence of EBV genomes in the previously reported case described in Table 2.

Because of the technical limitations of methods previously applied to the identification of donor-type relapses, the incidence of this phenomenon is not known. Also, donor relapses are now known to occur in both lymphocytic and myelocytic lineages, and neoplastic relapses can occur in cells of a different cell type. Prospective investigations are underway, utilizing restriction fragment length polymorphisms and careful phenotypic characterization of original and recurrent tumors in an effort to determine the incidence of this phenomenon and its biologic spectrum.

ACKNOWLEDGMENT

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