Restriction Fragment Length Polymorphisms as Markers of Engraftment in Allogeneic Marrow Transplantation

By Bruce R. Blazar, Harry T. Orr, Diane C. Arthur, John H. Kersey, and Alexandra H. Filipovich

We have used DNA hybridization techniques employing restriction fragment length polymorphisms (RFLPs) to quantitate the level of donor cell engraftment in bone marrow transplantation recipients. The genetic origin of the bone marrow cells and various peripheral blood populations was analyzed in 14 patients. We found at least one informative polymorphism for each donor-recipient pair. Additional markers of engraftment included cytogenetic analysis, HLA typing, and red cell typing. By DNA analysis, four patients had complete engraftment, five had partial engraftment, and five had no evidence of donor cell engraftment. In three cases, DNA analysis permitted detection of minor populations (5% to 10%) of donor or host cells. Eight of fourteen patients were evaluable for chimerism posttransplant by cytogenetic analysis. In five cases, cytogenetic results were completely concordant with DNA analyses. In two cases of apparent autologous recovery, as assessed using RFLPs, a small number of cells of donor karyotype was seen. In one other case, a small number of cells of host karyotype was not detected by RFLP studies. HLA typing in three partially engrafted patients was purely either of donor or host type. Red cell typing was discordant with DNA and/or cytogenetic results in four of eight cases. We conclude that DNA analysis at a limited number of informative genetic loci is useful for quantitating the degree of engraftment in multiple populations of nondividing cells following allogeneic bone marrow transplantation.

MATERIALS AND METHODS

Patient Population

The clinical characteristics of the recipients of transplants are summarized in Table 1. All patients received transplants at the University of Minnesota Hospitals between Nov 1981 and Oct 1983. The patients ranged in age from 0.6 to 23 years (median age, 7.4 years). Eight patients received transplants for nonmalignant diseases that included severe combined immune deficiency syndrome (SCID) three patients, Wiskott-Aldrich syndrome (WAS) two patients, and one patient each with Chediak-Higashi syndrome, common variable immunodeficiency, and mucopolysaccharidosis type I (MPS-I). Two of these patients (cases No. 6 and 7) received marrow from histocompatible siblings and six (cases No. 1 through 5 and 8) from partially mismatched donors following T cell depletion. The transplantation procedure and post-BMT management have been previously described. Six of the seven patients with underlying immunodeficiency were conditioned with cyclophosphamide (60 mg/kg/d for two days) and 7.5 gray (Gy) total body irradiation (TBI) delivered at a dose rate of 26 cGy/min. Two patients (cases No. 4 and 5) were further conditioned with intravenous procarbazine and antithymocyte globulin (ATG), as previously described. One patient with SCID (case No. 6) had no pre-BMT immunosuppression. The patient with MPS-I (case No. 7) received oral busulfan (16 mg/kg total dose) and cyclophosphamide (50 mg/kg/d for four days).

Six patients received transplants for leukemia. Four of these patients had acute lymphocytic leukemia (ALL) and one patient each had chronic myelogenous leukemia and acute nonlymphocytic leukemia (ANLL). These patients received chemotherapy consisting of cyclophosphamide (60 mg/kg/d for two days) or cytosine arabinoside (3 g/m² per dose for six doses) with single-dose (8.5 Gy at 0.26 cGy/min) or fractionated-dose (13.2 Gy at 10 cGy/min) TBI. One patient (case No. 13) was prepared for BMT with cyclophosphamide (60 mg/kg/d for two days), VM-26 (165 mg/m²/d for three days), cytosine arabinoside (300 mg/m²/d bolus infusion for three days), and TBI (8.5 Gy at 26 cGy/min).

All patients with leukemia were histocompatible with their donors. Graft-vs-host disease (GVHD) prophylaxis consisted of methotrexate, ATG, and prednisone in standard doses (cases No. 9 and 12) or ex vivo T cell depletion of the donor bone marrow. T cell depletion was accomplished by differential soybean agglutination and rosette depletion (cases No. 1 through 3), rosetting three times with neuraminidase-treated sheep erythrocytes (cases No. 4 and 5) or, more recently, with monoclonal antibody–ricin conjugates (cases

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USE OF RFLPs AS MARKERS OF ENGRAFTMENT

Table 1. Summary of Patient Characteristics

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Diagnosis</th>
<th>Donor Histocompatible</th>
<th>Conditioning Regimen*</th>
<th>GVHD Regimen†</th>
<th>Marrow Cell Dose (x 10^6/kg)</th>
<th>Current Status</th>
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<tr>
<td>1</td>
<td>UPN 237</td>
<td>14/F</td>
<td>CVID</td>
<td>No</td>
<td>Cy/TBI</td>
<td>SBA-E-E*</td>
<td>0.87 Alive d 850; transient improvement</td>
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<td>2</td>
<td>UPN 247</td>
<td>0.7/F</td>
<td>CHS</td>
<td>No</td>
<td>Cy/TBI</td>
<td>SBA-E-E*</td>
<td>1.09 Died d 68; CMV pneumonitis</td>
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<td>3</td>
<td>UPN 252</td>
<td>6.5/M</td>
<td>WAS</td>
<td>No</td>
<td>Cy/TBI</td>
<td>SBA-E-E*</td>
<td>0.50 Alive d 777; no improvement</td>
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<tr>
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<td>1.9/M</td>
<td>SCID</td>
<td>No</td>
<td>Cy/Pz/ATG/TBI</td>
<td>E^x x 3</td>
<td>1.20 Died d 221; accidental hemorrhage; improved immune function</td>
<td></td>
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<tr>
<td>5</td>
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<td>0.6/M</td>
<td>SCID</td>
<td>No</td>
<td>Cy/Pz/ATG/TBI</td>
<td>E^x x 3</td>
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<td>None</td>
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<td>7</td>
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<td>2/F</td>
<td>MPS-I</td>
<td>Yes</td>
<td>Cy/Busulfan</td>
<td>M-A-P</td>
<td>2.23 Alive d 440; clinical improvement</td>
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<tr>
<td>8</td>
<td>UPN 332</td>
<td>7.8/M</td>
<td>WAS</td>
<td>No</td>
<td>Cy/ATG/TBI</td>
<td>TUT-ricin</td>
<td>1.69 Alive d 440; no improvement</td>
<td></td>
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<tr>
<td>9</td>
<td>UPN 172</td>
<td>23/M</td>
<td>CML</td>
<td>Yes</td>
<td>Cy/AraC/Fx TBI</td>
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<tr>
<td>10</td>
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<td>21/M</td>
<td>ANLL</td>
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<tr>
<td>11</td>
<td>UPN 344</td>
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<td>Yes</td>
<td>Cy/Fx TBI</td>
<td>TUT-ricin</td>
<td>1.00 Died d 309; relapse d 167</td>
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<tr>
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<td>ALL</td>
<td>Yes</td>
<td>Cy/Fx TBI</td>
<td>M-A-P</td>
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</tr>
<tr>
<td>13</td>
<td>UPN 373</td>
<td>7/M</td>
<td>ALL</td>
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<td>1.53 Alive d 240; relapse d 177</td>
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<td>14</td>
<td>UPN 382</td>
<td>13/M</td>
<td>ALL</td>
<td>Yes</td>
<td>AraC/TBI</td>
<td>TUT-ricin</td>
<td>2.08 Alive d 220; relapse d 75</td>
<td></td>
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</tbody>
</table>

Abbreviations: CVID, common variable immune deficiency; CHS, Chediak-Higashi syndrome; WAS, Wiskott-Aldrich syndrome; SCID, severe combined immune deficiency; MPS-I, mucopolysaccharidosis, type I; CML, chronic myelogenous leukemia; ANLL, acute nonlymphoblastic leukemia; ALL, acute lymphoblastic leukemia.

*Cy, cyclophosphamide; TBI, total body irradiation; Pz, procarbazine; ATG, antithymocyte globulin; Fx TBI, fractionated total body irradiation.
†SBA-E-E*, differential soybean agglutination technique; E^x x 3, separation by rosetting three times with neuraminidase-treated sheep erythrocytes; TUT-ricin, depletion by monoclonal antibody ricin conjugates; GVHD, graft-versus-host disease; M-A-P, methotrexate, antithymocyte globulin, and prednisone.

No. 8, 10, 11, 13, and 14), as previously described. Marrow cell doses ranged from 0.69 to 2.08 x 10^6 nucleated cells per kilogram body weight for patients receiving T cell-depleted donor marrow and 2.00 to 3.36 x 10^6 nucleated cells per kilogram body weight for patients receiving untreated bone marrow.

**RFLP Analysis**

Cell populations studied. DNA was extracted from heparinized peripheral blood cells or frozen peripheral blood mononuclear cells collected from donor and recipient pre-BMT. When adequate pre-BMT samples were unavailable, fibroblast cultures, obtained by standard methods, served as a source of DNA of the recipient genotype. Post-BMT, at varying intervals, 10 to 60 mL of heparinized peripheral blood cells were separated into mononuclear cells and neutrophils by Ficoll-Hypaque density gradient centrifugation. In one instance (case No. 8), peripheral blood cells were further fractionated into T lymphocytes and non-T cells by neuraminidase-treated sheep erythrocytes, as previously described. The T cells were 95% rosette positive, while the non-T cells were 2% rosette positive. Infrequently, cell numbers were inadequate to permit fractionation, and therefore, unseparated blood was analyzed. Bone marrow specimens were not fractionated prior to DNA extraction. As few as 10^6 cells provided sufficient DNA for analysis.

**Preparation of high-molecular weight DNA.** High-molecular weight DNA was isolated by the method of Kunkel et al. Aliquots of 3 to 12 µg of purified DNA (quantitated by spectrophotometric methods) were cleaved with restriction endonucleases (EcoRI, HindIII, or TaqI) according to manufacturer's (Bethesda Research Labs, Gaithersburg, Md) specifications; further DNA quantitation was obtained by electrophoresis of 10% of the digested sample alongside 1 µg of predigested genomic DNA. Digested samples were electrophoresed in either 0.5% (EcoRI-digested DNA) or 0.7% (HindIII- or TaqI-digested DNA) agarose, and then transferred by the Southern blotting method on Zetabind membranes (AMF, Cuno-Meridan, Conn). Hybridization was carried out in the presence of 50% formamide at 42 °C (probes B7 cDNA, 2A.1, or pAW101) or 47 °C (probe pDP34). The description of these probes are found below. All probes were radiolabeled by nick translation, with [32P]-[a]-dCTP (Amersham-Searle, Elkhart, Ind), to a specific activity of 0.8 to 3.0 x 10^6 cpm per microgram of DNA. Nonspecifically bound radioactivity was removed by washing the membrane at increasing stringency with a final wash in 0.1 x SSC (1 x SSC is 0.15 mol/L NaCl, 0.015 mol/L Na citrate) and 0.1% sodium dodecyl sulfate at 42 °C (probes B7 cDNA, 2A.1), 55 °C (pDP34), or 65 °C (pAW101). Hybridization was visualized by exposing the filters for two to ten days at −80 °C on Kodak XAR5 (Eastman-Kodak, Rochester, NY) with intensifying screens.

**Quantitation of Engraftment.** The degree of engraftment was quantitated by gel scanning of autoradiograms exposed for two to ten days depending on the initial 24-hour autoradiogram. The films
were exposed for a variable period of time to optimize detection of small numbers of donor or host cells. The quantitation of the degree of engraftment in post-BMT samples was adjusted for differences in the relative densitometry readings of the allelic patterns of the donor and pre-BMT host samples (which were included on the autoradiograms of post-BMT samples). Each autoradiogram was scanned twice on a Cliniscan (Helena Laboratories, Beaumont, Tex) using a neutral density filter scanning at 520 nm with a slit width of 8.0 nm, and the results of the two scans were averaged. In instances in which incomplete engraftment was documented by gel scanning, the percentage of donor cells was expressed to the nearest 5%.

Other Markers of Engraftment

Cytogenetic analyses, HLA typing, and red cell phenotyping. For cytogenetic analysis, metaphase cells were obtained from direct preparations and short-term unstimulated cultures of bone marrow and from phytohemagglutinin-stimulated methotrexate-synchronized peripheral blood cells. Pre-BMT, in all cases, donor and recipient bone marrow samples were examined using G-banding for constitutional karyotype and leukemia-associated chromosomal alterations. In addition, peripheral blood samples from same-sex donor and recipient pairs were studied with Q-banding for polymorphic differences. Post-BMT, a minimum of 20 metaphases were karyotyped (whenever possible) and analyzed for evidence of donor sex chromosomes or polymorphisms as well as chromosomal aberrations characteristic of relapse in patients transplanted for leukemia. Cytogenetic analysis was performed within four days of DNA analysis in five cases (cases No. 7, and 11 through 14) and less than three weeks prior to DNA analysis in four others: case No. 1 (day 650, bone marrow sample only), case No. 2 (day 62), case No. 5 (day 57), and case No. 8 (day 166).

HLA typing was based on a two-stage microcytotoxicity assay. HLA-A, -B, and -C typing was performed on peripheral blood mononuclear cells, while HLA-D, -DQ, -DS, and -DR typing was assessed using isolated B lymphocytes. HLA typing was analyzed within three weeks of RFLP analysis in five of six cases. Case No. 1 had HLA typing both three and six months prior to RFLP analysis.

Red cell phenotyping was accomplished by serotyping with standard antigens. Early post-BMT, the reticulocyte-enriched fraction of Technology, Cambridge) contains a 2.2-kb genomic insert to the previously described probe pDP31. This probe hybridizes with two fragments, depending on the HLA-A typing of the individual tested. Probe pAW101 (generously supplied by Dr. Ray White, University of Utah, Salt Lake City, and the Howard Hughes Medical Center, Utah) contains a 5-kb genomic insert in the plasmid pBR322. This probe hybridizes to highly polymorphic EcoRI-generated fragments derived from allelic sequences located on chromosome 14. More than ten different alleles at a single locus on chromosome 14 have been described.

For sex-mismatched pairs, the probe pDP34 was used. Following TaqI digestion of genomic DNA, this probe detects two allelic X-specific fragments in females of 10.6 and 11.8 kb, respectively, and a Y-specific fragment of 14.6 kb, in addition to either one of the X-specific fragments in male individuals. For same-sex HLA-mismatched patients, we first used either the probe B7 cDNA or 2A1. One patient (case No. 3), who was identical with his donor at the HLA-B locus and partially matched at the HLA-A locus, was initially screened using the B7 cDNA probe. No polymorphisms were identified. Rehybridization of the filter with pAW101 did reveal a polymorphic marker, and hence, no further screening was performed. For same-sex HLA-identical patients, the initial screening was performed with pAW101.

Sensitivity of Southern Blotting Analysis of Engraftment

In a mixing experiment designed to determine the sensitivity of the detection of a donor- or host-specific fragment, control DNA samples from a male and a female were mixed in varying proportions ranging from 2.5% to 25% ratios of male/female DNA. The DNA sample was then digested with TaqI and incubated with a radiolabeled probe, pDP34, as described above. The presence of a 14.6-kb male-specific fragment would be indicative of the sensitivity level of this system. An initial autoradiogram was exposed for 24 hours with two intensifying screens. The 14.6-kb Y-specific fragment was detected only in the 12-µg DNA mixture containing 25% male-derived DNA. However, after a nine-day exposure (as shown in Fig 1), the 14.6-kb fragment was seen in all 6- and 12-µg samples containing 2.5% or more male-derived DNA. In smaller samples in which a total of only 3 µg of DNA was available for restriction enzyme digestion, a minimum of 10% male-derived DNA was required. In a subsequent experiment using 12 µg of DNA in concentrations ranging from 0.5% to 50% male-derived DNA, with the remainder of the sample composed of female-derived DNA, a 1% detection level was achieved after ten days of exposure of the autoradiogram (data not shown). We conclude that 10% of a minor population of donor- or host-derived DNA can be routinely detected if sufficient DNA is available for analysis and the autoradiogram is exposed to permit optimal detection of low levels of DNA of a different genotype.

Results of Polymorphism Screening Using a Limited Panel of Probes

DNA analysis was maximally informative when both the donor and the recipient had unique fragments (cases No. 2, 4, 5, 9, 10, 12, and 13, Table 2; Fig 2). A single unique donor or host fragment could provide maximal information in certain situations (cases No. 1, 6, 7, and 14).
Kb 1 2 3 4 5 6 7 8 9 10
14.6 —
11.8 —

Fig 1. Sensitivity of Southern blotting analysis. In an experiment designed to test the sensitivity of DNA analysis by Southern blotting, varying total quantities of DNA (3, 6, or 12 μg) consisting of 2.5% to 25% of DNA obtained from a control male (donor) were mixed with 75% to 97.5% of DNA obtained from a control female (recipient). The DNA was digested with restriction enzyme TaqI and hybridized with the probe pDP34. Lane 1, 3 μg total DNA, 5.0% donor; lane 2, 3 μg total DNA, 10.0% donor; lane 3, 3 μg total DNA, 25.0% donor; lane 4, 6 μg total DNA, 25.0% donor; lane 5, 6 μg total DNA, 5.0% donor; lane 6, 6 μg total DNA, 10.0% donor; lane 7, 6 μg total DNA, 25.0% donor; lane 8, 12 μg total DNA, 2.5% donor; lane 9, 12 μg total DNA, 5.0% donor; lane 10, 12 μg total DNA, 10.0% donor. The recipient is homozygous for the 11.8-kb allele. The 14.6-kb donor allele is visualized in all lanes except lane 1. Therefore, 2.5% donor DNA can be detected in 97.5% recipient DNA when 6 or 12 μg of total DNA is digested. However, 10.0% donor DNA is required for visualization of the 14.6-kb allele when 3 μg of total DNA is digested.

A summary of the chimerism data is listed in Table 2. By DNA analysis, four patients (cases No. 9, 10, 12, and 13) were initially noted to have complete engraftment of all cell populations tested.

Only case No. 13 was analyzable for cytogenetic evidence of engraftment. Initially, no host cells were detectable by either technique. Later, on days 28 and 74, a small proportion of cells in metaphase were of host karyotype. These cells had a clonal chromosomal abnormality characteristic of this patient’s original leukemic population, and therefore, represented subclinical leukemic relapse.

Five patients (cases No. 4 through 7 and 14) had partial engraftment as determined by DNA analyses (eg, Fig 3). Cytogenetic analysis was confirmatory in each of the three analyzable cases (cases No. 5, 7, and 14).

Five patients (cases No. 1 through 3, 8, and 11) were noted to have autologous recovery by DNA analysis. Cytogenetic analysis was informative in four cases (cases No. 1, 2, 8, and 11) post-BMT. Two of these patients (cases No. 8 and 11) experienced graft rejection. At the time of the DNA analyses, cells in metaphase were of host karyotype exclusively. In the remaining two patients (cases No. 1 and 2, shown in Fig 4), a small percentage of cells in metaphase were of donor type.

HLA typing was discordant with DNA analyses and/or cytogenetic analyses in the three cases of partial engraftment (cases No. 1, 4, and 5), but was concordant in one case of autologous recovery (case No. 3). Of additional note, in three cases (cases No. 1, 2, and 8), the results of both HLA class I antigen typing and HLA-D typing were discordant with the HLA-DQ and/or HLA-DR typing of the same patient, and therefore, the results were difficult to interpret.

Red cell typing was feasible in eight recipients (cases No. 1, 3, 4, and 6 through 10) post-BMT. In four cases (No. 1, 3, 8, and 10), red cell phenotyping was discrepant with the other markers of engraftment at least at one time point.

Clinical Course of Engrafted Patients

Eleven patients (cases No. 1 through 8, 10, 11, and 14) had at least one parameter consistent with partial engraftment at some period post-BMT. These patients have provided us with an opportunity to study the clinical and/or laboratory course of partially engrafted patients. All eight of the patients with nonmalignant disorders (cases No. 1 through 8) had partial engraftment by DNA analysis, cytogenetic analysis, and/or red cell typing. In six cases (No. 1 and 4 through 8) of the eight patients, two or more of these parameters were consistent with partial engraftment of the recipient. Five (cases No. 1 and 4 through 7) of seven evaluable cases (No. 1 and 3 through 8) showed clinical improvement. Two patients (cases No. 3 and 8) had only red cell engraftment and had no evidence of clinical improvement posttransplant. One of these patients (case No. 8) was further studied for isolated T cell engraftment. No evidence of T cell or non-T cell engraftment was present (see Discussion). One patient (case No. 2) died prior to day 100 and was not evaluable for improved immune function. Therefore, in most of these patients with nonmalignant disorders, clinical and/or laboratory improvement accompanied partial engraftment of donor cells. Further details are being reported elsewhere (A.H.F., unpublished observations June, 1984).

All three patients (cases No. 10, 11, and 14) with leukemia who had partial engraftment relapsed post-BMT. Of the remaining three patients (cases No. 9, 12, and 13) with leukemia who had complete engraftment by DNA analysis, one patient (case No. 9) has remained relapse-free for longer than 2½ years, one patient (case No. 12) was relapse-free at
The percentage of donor cells was quantitated by gel scanning techniques when partial engraftment was noted by the appearance of mixed donor and host genotype pattern. tMetaphase cells are identified as donor/recipient. Red cell typing was characterized as donor/recipient or D/R mixed-field agglutination representing populations of donor and recipient cells. §Post-BMT HLA-DQ and/or HLA-DR typing discordant with HLA-A, -B, -C, or -D typing of the recipient, precluding determination of mononuclear cells as of donor or host origin. Blood mononuclear cells were further separated into T cells and non-T cell fractions. The mononuclear cell layer contained 90% circulating blasts; the neutrophil layer contained no blasts; the bone marrow aspirate contained 36% blasts.

The time of death (100 days) post-BMT, and the third patient (case No. 13) had a clinical relapse 177 days post-BMT.

For eight patients (cases No. 1, 2, 7, 8, 10, 11, 13, and 14) in whom serial studies were performed, a limited assessment of graft stability is possible. Three patients (cases No. 8, 10, and 11) had abrupt changes in engraftment status consisting of graft rejection (cases No. 8 and 11) or leukemia relapse (case No. 10). The remaining patients experienced either slight increases or decreases in the percentage of donor type cells.

**DISCUSSION**

We have compared the utility of RFLPs with cytogenetic analysis, red cell typing, and HLA typing as methods for studying allogeneic marrow engraftment in 14 BMT recipients. We identified useful polymorphisms in each donor–recipient pair pre-BMT with a limited panel of probes and restriction endonucleases. The use of a small panel of probes offers several advantages when compared to larger panels: the screening procedure is efficient in cost, time, and can be performed with a limited sample quantity. However, maximally informative polymorphisms may be more difficult to detect. A more efficient system could be developed by using only one or two restriction endonucleases that have a high likelihood of generating highly polymorphic sequences. Two restriction endonucleases—TaqI or MspI—digest DNA which contains the common dimer sequence CpG. These restriction sites have been shown to have a high frequency of polymorphism in human DNA. By using multiple radiolabeled probes in the initial hybridization reaction or by
reprobing filters hybridized with a single radiolabeled probe, the screening procedure could be made more efficient, particularly for same-sex HLA-identical donor-recipient combinations. Moreover, the use of probes hybridizing to hypervariable "minisatellite" regions of DNA as recently described by Jeffreys et al would permit the detection of a polymorphic marker in virtually all donor-recipient pairs with a single screening filter.26

Our patient population was, in part, selected to provide patients who already had, or would prove to have, incomplete

Table 3. DNA Analysis

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient No.</th>
<th>Probe</th>
<th>Donor Fragments</th>
<th>Host Fragments</th>
<th>Post-BMT Fragments</th>
<th>Informative Alleles</th>
<th>Interpretation of Post-BMT RFLP Results</th>
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<tr>
<td>1</td>
<td>UPN 237</td>
<td>2A.1</td>
<td>5.1, 4.7</td>
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<td>pDP34</td>
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<td>11.8, 10.6</td>
<td>+</td>
<td>Autologous recovery</td>
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<td>+</td>
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<td>UPN 332</td>
<td>pDP34</td>
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<td>Autologous recovery*</td>
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<td>pDP34</td>
<td>11.8, 10.6</td>
<td>14.6/—†</td>
<td>11.8, 10.6</td>
<td>+</td>
<td>Complete engraftment</td>
</tr>
<tr>
<td>10</td>
<td>UPN 338</td>
<td>pAW101</td>
<td>A, B</td>
<td>NA</td>
<td>A, B</td>
<td>Complete engraftment; relapse in host-type cells</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>UPN 344</td>
<td>pDP34</td>
<td>NA†</td>
<td>14.6, 11.8</td>
<td>14.6, 11.8</td>
<td>+</td>
<td>Autologous recovery*</td>
</tr>
<tr>
<td>12</td>
<td>UPN 367</td>
<td>pDP34</td>
<td>11.8, 10.6</td>
<td>14.6/—†</td>
<td>11.8, 10.6</td>
<td>+</td>
<td>Complete engraftment</td>
</tr>
<tr>
<td>13</td>
<td>UPN 373</td>
<td>pDP34</td>
<td>11.8, 10.6</td>
<td>14.6, 10.6</td>
<td>11.8, 10.6</td>
<td>+</td>
<td>Complete engraftment</td>
</tr>
<tr>
<td>14</td>
<td>UPN 382</td>
<td>pDP34</td>
<td>10.6</td>
<td>14.6, 10.6</td>
<td>14.6, 10.6</td>
<td>+</td>
<td>Mixed chimerism</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

Allelic fragments are given in kb for probes pDP34 and 2A.1 or named by letters (probes b7cDNA and pAW101): A, B, C, D represent the largest to the smallest fragments for each donor-recipient pair.

*Based on consistent gel scanning data, although 10% engraftment would not have been detectable.
†DNA samples were not available for study but presumably contain the 14.6-kb Taq male-specific fragment.
‡DNA sample was not available for study but presumably lacks the 14.6-kb Taq male-specific fragment.

Fig 2. Leukemia relapse post-grafting: determination of cell of origin (case No. 10). DNA analysis of this patient's leukemia relapse has demonstrated that relapse occurred in host-type cells. DNA from the donor pretransplant and the recipient posttransplant was digested with the restriction enzyme EcoRI and then hybridized with the probe pAW101 by Southern blotting techniques. Lane 1, donor pre-BMT; lane 2, recipient unseparated peripheral blood, day 82; lane 3, recipient unseparated peripheral blood, day 91; lane 4, recipient unseparated peripheral blood, day 142; lane 5, recipient neutrophils, day 142; lane 6, recipient peripheral blood mononuclear cells, day 142; lane 7, bone marrow (36% blasts), day 167; lane 8, peripheral blood mononuclear cells (90% blasts), day 167; lane 9, recipient unseparated peripheral blood, day 167; lane 10, recipient neutrophils (0% blasts), day 167. Although no pre-BMT recipient DNA was available for analysis, the recipient genotype was deduced from the donor genotype and from the changed autoradiographic pattern of the recipient at the time of leukemia relapse. The arrow denotes the location of the presumed host-specific fragment. The quantitation of donor cell engraftment correlated with the percentage of blast cells in each population.

Fig 3. Differential engraftment of fractionated peripheral blood cells (case No. 14). DNA was obtained from the donor (female) and recipient (male) prior to BMT. Following digestion with TaqI and hybridization with the probe pDP34, a 14.6-kb host-specific allele was detected. Post-BMT peripheral blood was fractionated (day 25) and analyzed by Southern blotting. Lane 1, donor pre-BMT; lane 2, recipient pre-BMT; lane 3, recipient mononuclear cells, day 15; lane 4, recipient pre-BMT; lane 5, recipient mononuclear cells, day 15; lane 6, recipient mononuclear cells, day 25; lane 7, recipient neutrophils, day 25. The donor had a 14.6-kb Y-specific allele and an X-specific allele of 11.8 DNA on day 25, while the neutrophils on day 26 were entirely of donor origin. Leukemia relapse occurred on day 75. This recipient received a transplant in early relapse and received T cell-depleted donor marrow. Post-BMT, the recipient's mononuclear cells were of mixed donor and host origin (85% donor DNA, day 15; 95% donor DNA) at day 25 post-BMT.
engraftment or graft rejection. Ten patients (six of whom were histoincompatible with their donors) received marrow depleted of T lymphocytes. Both incomplete engraftment and marrow rejection have been noted in BMT recipients of T cell-depleted and/or histoincompatible donor bone marrow.7,23,33 Two of the remaining patients were at higher risk for incomplete engraftment as a consequence of their pre-BMT conditioning regimen. One patient with SCIDS and absent natural killer cell function (case No. 6) received histocompatible marrow without pre-BMT immunosuppres-

Fig 4. Apparent autologous recovery by DNA analysis (case No. 2). This 8-month-old girl with Chediak-Higashi syndrome received histoincompatible T cell-depleted bone marrow from her father. DNA obtained from the donor pre-BMT and the recipient pre- and post-BMT was digested with TaqI and hybridized with the probe pDP34 by Southern blotting methods. Appearance of the Y-specific 14.6-kb donor allele would be indicative of marrow engraftment. Post-BMT, there was no evidence of donor cell engraftment by DNA analysis. Lane 1, donor pre-BMT; lane 2, recipient pre-BMT; lane 3, recipient peripheral blood mononuclear cells, day 11; lane 4, recipient peripheral blood mononuclear cells, day 62.

In this setting, engraftment of isolated cell lineages has been noted.34 We speculate that this patient's partial neutrophil engraftment, which occurred despite the administration of no pre-BMT myeloblastolysis, may be related to this patient's profound immunosuppression, a potentially defective myeloid precursor cell, as well as his post-BMT GVHD prophylactic immunosuppressive regimen. Although this patient did not have neutropenia at the time of BMT (age, 10 months), a patient with combined immunodeficiency with predominant T cell defect and neutropenia has been recently reported.35 Neutropenia occurred at 1 year of age, and may have been the result of a defective myeloid precursor. The other patient with a metabolic storage disease (case No. 7) did not receive any radiotherapy. Instead, this patient was conditioned with cyclophosphamide and high-dose busulfan, a regimen which we and others have noted to be insufficient to ensure uniform complete engraftment of all cell lines in patients with nonmalignant disorders.36,37 Unique patient No. (UPN) 367 (case No. 12) was selected for DNA analysis post-BMT to determine if the severe leukopenia (leukocyte count <0.3 x 10^9 per liter) noted 39 days post-BMT represented graft rejection.

Complete engraftment of all cell populations by DNA analysis, cytogenetic studies, HLA typing, and red cell typing was documented in only three patients. The remaining 11 patients served as a basis for comparative analysis of the various available methodologies. DNA analysis was consistent with complete engraftment in four of the patients. One of these patients (case No. 10) had mixed red cell engraftment. Of five patients with partial engraftment by RFLP analysis, in two (cases No. 4 and 5) HLA typing was the only marker that was not consistent with partial engraftment. No evidence of donor cell engraftment was present by RFLP analysis in five cases. In two of these cases, a small percentage of donor red cells was found 166 and 491 days post-BMT, while in two other cases, a small number of cells in metaphase were of donor origin. In the remaining instance, both DNA analysis and cytogenetic analysis indicated that the donor graft was no longer present post-BMT.

DNA analysis in this group of patients has permitted detection of 5% to 10% donor or host cells in three patients. In other instances, the fragments visualized on the autoradiograms were of sufficient intensity to predict that 1% to 5% of donor cells would have been detectable (eg, Fig 4). This level of sensitivity is consistent with in vitro DNA mixture experiments we and others have performed.27,38 In the cases in which DNA analysis did not demonstrate engraftment at these levels, we speculate that the higher mitotic rate of donor cells (with or without additional mitogenic stimuli), especially early post-BMT, may account for the discrepancies. Lawler et al have summarized their experience with the cytogenetic analysis of 45 sex-mismatched allogeneic BMT patients.39 Peripheral blood cells were stimulated with one of three mitogenic agents (phytohemagglutinin, lipopolysaccharide, or pokeweed mitogen), or left unstimulated. Peripheral blood mononuclear cells stimulated with phytohemagglutinin, which were entirely of donor origin, were noted to be of mixed donor and host origin when a different mitogenic agent was used (lipopolysaccharide or pokeweed mitogen). In the same study, unstimulated mononuclear cells documented a single metaphase of recipient type at a time when phytohemagglutinin stimulation revealed six metaphases of donor type only. These apparent differences in the mitogenic responsiveness of donor and recipient cells post-BMT and the low statistical probability of detecting a minor population of either donor or host cells (which is dependent on the level of the mixed chimerism as well as the number of metaphases analyzed)40 may partially or wholly account for a portion of these results.

An additional explanation for the discordant results in two cases is the differential mitotic rate of individual cell populations studied. Bone marrow cells (including stem cells, myeloblasts, and erythroblasts) have a high rate of spontaneous mitosis. Peripheral blood cells that are likely to enter mitosis are circulating progenitor cells or phytohemagglutinin-responsive mononuclear cells (predominantly T cells). In vitro culturing of the same samples prior to karyotypic analysis may also contribute to the observed discrepancies with RFLP analysis.

The true physiologic level of engraftment and graft stability could not be definitively assessed in this preliminary study. Persistence of endogenous cell populations41-43 may occur more frequently than previously believed. In this study, we have found a high percentage of incomplete engraftment in our patient population as assessed by DNA analysis using
USE OF RFLPs AS MARKERS OF ENGRAFTMENT

density quantitation of autoradiograms. However, caution must be exercised in the quantitative interpretation of the percentage of host and donor cells as assessed by simple autoradiographic densitometry. Variation in the efficiency of DNA transfer, degree of shearing, and/or DNA degradation in the sample nonspecific hybridization of the DNA probe to the filter membrane and nonlinearity of the intensity of the fragments on the autoradiogram (particularly at the extremes of exposure) can all contribute to errors in accurately documenting the level of donor cell engraftment. In comparisons with conventional methodologies used for documenting donor cell engraftment, HLA typing was found to be inadequate in documenting cases of partial engraftment. Furthermore, the interpretation of HLA typing post-BMT was difficult in three cases (cases No. 1, 2, and 8) in which the results of HLA-DQ and/or -DR typing were contradictory to the results of class I antigen typing and typing of other class II antigens. In addition to documenting donor cell engraftment with RFLPs, Southern blotting techniques are useful in determining the donor or host origin of patients who have post-BMT leukemia (eg, Fig 3), or as previously described for leukemia of host origin or posttransplant lymphoma.

In summary, we have shown that DNA analysis is of value in detecting engraftment in patients who lack cytogentic markers or whose post-BMT cells are mitotically inactive. In addition, we have demonstrated that DNA analysis is useful in quantitating engraftment in separate cell populations, documenting the cell origin of leukemia relapse post-BMT, and assessing patients for graft rejection. With current techniques, DNA analysis is sensitive to a level of 10% or less. We conclude that DNA analysis should be of continuing importance in the routine assessment of chimerism in allogeneic marrow transplant recipients.

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REFERENCES

Restriction fragment length polymorphisms as markers of engraftment in allogeneic marrow transplantation

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