Myeloid and Lymphoid Chimerism After T-Cell–Depleted Bone Marrow Transplantation: Evaluation of Conditioning Regimens Using the Polymerase Chain Reaction to Amplify Human Minisatellite Regions of Genomic DNA


Determining both myeloid and lymphoid chimerism after T-cell–depleted allogeneic bone marrow transplantation (BMT) could be helpful in the understanding of the biology of engraftment and could provide a rational method of assessing the ability of different conditioning regimens to promote engraftment. We prospectively investigated the role of different pretransplant conditioning regimens in 29 leukemic patients post-BMT by assessing myeloid and T-cell chimerism using a rapid and sensitive polymerase chain reaction (PCR) method. Minisatellites are hypervariable regions of DNA consisting of tandem repeats of a core nucleotide sequence, and allelic polymorphism results from differences in the number of the repeats. We used this variation to distinguish between donor and recipient cells post-BMT. Seventeen patients (9 sibling and 8 unrelated donors) received conditioning with hyperfractionated total body irradiation (TBI), thiotepa, and cyclophosphamide (Cy). Of the other 12 patients (all sibling donors), 11 received TBI plus Cy plus another agent: VP16, carboplatinum, or AZQ. One patient received TBI plus thiotepa plus VP16. All but one of the patients studied received marrow from HLA-identical donors. PCR analysis confirmed donor lymphoid engraftment within 8 days of transplant in six of six patients studied. All granulocyte DNA was of donor origin within the first 4 weeks of transplant, regardless of the conditioning regimen. The day +28 T cells were exclusively of donor origin in 14 of 17 patients who received TBI plus thiotepa plus Cy, but were mixed chimeric in 10 of 12 patients who received other conditioning regimens (P < .001). Early graft rejection was seen in one unrelated transplant recipient conditioned with TBI plus thiotepa plus Cy. Late graft failure was observed in 3 of 12 patients with mixed T-cell chimerism and in none of 16 patients with full donor chimerism at day +28. However, 5 of 16 patients who had complete T-cell chimerism at day +28 developed acute graft-versus-host disease (GVHD), whereas no patient with mixed chimerism had acute GVHD. Our results indicate that minisatellite PCR is a rapid and sensitive method for assessing chimerism post-BMT, that the donor T cells are important for consistent durable engraftment, and that TBI plus thiotepa plus Cy may be superior to the other regimens studied in inducing full donor chimerism. Larger numbers and longer follow-up are necessary to confirm these data and also to assess the relationship between complete donor T-cell chimerism and leukemia-free survival.

© 1992 by The American Society of Hematology.

Bone marrow transplantation (BMT) is the treatment of choice for many patients with hematologic malignancies. The use of this treatment modality is based on the assumption that the high-dose chemoradiotherapy used as pretransplant conditioning therapy will not only eradicate the malignant clone but will also result in a degree of host immunosuppression sufficient to prevent immunologically mediated graft rejection by the host. In vitro T-cell depletion of the donor marrow has been used successfully to prevent graft-versus-host disease (GVHD) after allogeneic BMT, but can be associated with an increased incidence of both graft failure and leukemia relapse. Before the introduction of T-cell depletion, it was generally considered that the conditioning regimens used in patients with leukemia such as cyclophosphamide (Cy) and total body irradiation (TBI) were “ablative,” with full donor chimerism being regularly observed as long as the patients remained in remission. More recently, it has been shown that mixed chimerism is more common after T-cell–depleted BMT and that it may predispose to graft rejection and leukemia relapse.1,2

Several techniques have been used to evaluate chimerism after allogeneic BMT. Methods used to detect mixed chimerism have included red blood cell (RBC) polymorphisms, cytotagentic analyses, and fluorescent in situ hybridization.3–5 These techniques can be of limited value due to limited degree of polymorphism, poor sensitivity, RBC transfusions, or the requirement for a donor and recipient that are sex-mismatched. More recently, chimerism has been studied with DNA techniques using restriction fragment length polymorphisms (RFLP), which are created by either the loss or gain of a restriction enzyme cleavage site or by the insertion or deletion of DNA between restriction sites. The former are generally 2 allele polymorphisms; the latter are often multiallelic if the insertions or deletions result in the formation of human minisatellites or variable number of tandem repeats (VNTR) regions of the genome.6 These minisatellite or VNTR loci are inherited in a Mendelian manner and have been useful in assessing chimerism post-BMT. Important limitations to conventional RFLP analysis post-BMT are the requirement for at least 10^6 cells to extract sufficient DNA for conventional Southern blot analysis and its lack of sensitivity in detecting minor cell populations. The introduction of the polymerase chain reaction7 (PCR) as a method for rapid amplification of human minisatellite regions has provided a powerful tool in assessing chimerism. By using PCR primers that flank the minisatellite loci, the whole allele is amplified and, therefore, the size of the PCR product is determined by the number of tandem repeats.8 The main advantage of a PCR-based method is enhanced sensitivity, which allows...
for the detection of minor populations of donor or recipient cells. In addition, PCR permits analysis from a small number of cells, thus allowing analysis of engraftment kinetics before there is morphologic evidence of engraftment.

This report describes the application of PCR to engraftment analysis after T-cell-depleted allogeneic BMT. We attempt to address three main issues: (1) the kinetics of engraftment in the early period post-BMT; (2) the documentation of chimerism in cells of both the myeloid and T-cell lineage; and (3) the impact of the conditioning regimen on chimerism, graft failure, and GVHD.

MATERIALS AND METHODS

Patients. Twenty-nine patients with acute or chronic leukemia undergoing T-cell-depleted allogeneic BMT were studied. Disease status, donor/recipient age and sex, donor type, method of T-cell depletion, and conditioning regimen are shown in Table 1. Twenty-eight donor-recipient pairs (21 sibling and 7 unrelated) were matched at the human leukocyte antigen (HLA) A, B and DR loci; one unrelated donor-recipient pair (unique patient number [UPN] 1152) was HLA A and B identical, but was mismatched for one DR locus. Clinical protocols were approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center and transplants were performed with the informed consent of the patients.

Transplant conditioning regimens. Seventeen patients (9 sibling and 8 unrelated donors) received conditioning with hyperfractionated total body irradiation 12 × 125 cGy (TBI) plus thiotepa 10 mg/kg plus cyclophosphamide 120 mg/kg (Cy). Of the other 12 patients (all sibling donors), 11 received TBI plus Cy plus another agent (VP16 at 750 mg/m², carboplatinum at 2 g/m², or aziridinylbenzoquinone [AZQ] at 196 mg/m²) based on the disease or stage of disease under treatment. One patient with compromised cardiac function received TBI plus thiotepa plus VP16 without Cy at the dose levels detailed above. Thirteen patients received antithymocyte globulin (ATG) at 15 mg/kg/d on alternate days from day +5 to +19 as additional graft rejection prophylaxis. Recipients of sibling transplants before December 1991 received ATG if the donor or recipient age was more than 29 years. In vitro T-cell depletion was performed using sheep agglutinin (SBA) to produce a SBA− marrow fraction. This was further T-cell depleted with either rosetting with sheep RBCs to give a SBA-E− marrow.

Table 1. Clinical Parameters of Patients Who Received T-Cell-Depleted BMT Together With the Results of the Day +28 Chimerism Studies

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>Recipient Age/Sex</th>
<th>Donor Age/Sex</th>
<th>ATG Post BMT</th>
<th>T-Cell Depletion</th>
<th>Donor Type</th>
<th>Conditioning Regimen</th>
<th>Chimeric Status on Day +28</th>
<th>Acute GVHD</th>
<th>Graft Failure</th>
<th>Present Status (mo post-BMT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1058</td>
<td>AML, 1</td>
<td>5/M 11/F</td>
<td>11/F E− Sibling</td>
<td>TBI + VP16 + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>DFS, 19+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1072</td>
<td>AML, 1</td>
<td>34/M 28/F</td>
<td>28/F CD4− Sibling</td>
<td>TBI + VP16 + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 10-Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1113</td>
<td>AML, 1</td>
<td>27/F 25/M</td>
<td>25/M E− Sibling</td>
<td>TBI + VP16 + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 8-Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1150</td>
<td>AML, 1</td>
<td>42/F 49/Y</td>
<td>49/Y CD4− Sibling</td>
<td>TBI + VP16 + Cy</td>
<td>Donor Mixed</td>
<td>Grade 3</td>
<td>6-GVHD/IP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1160</td>
<td>AML, 2</td>
<td>40/F 54/M</td>
<td>54/M E− Sibling</td>
<td>TBI + VP16 + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 5-Lymph</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1161</td>
<td>AML, 1</td>
<td>52/F 44/M</td>
<td>44/M E− Sibling</td>
<td>TBI + VP16 + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 8-Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1158</td>
<td>AML, 1</td>
<td>14/F 18/M</td>
<td>18/M E− Sibling</td>
<td>TBI + VP16 + thiotepa + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>DFS, 8+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1176</td>
<td>AML-Ref</td>
<td>56/F 49/M</td>
<td>49/M E− Sibling</td>
<td>TBI + AZQ + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 3-GF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1187</td>
<td>AML-Ref</td>
<td>39/M 48/F</td>
<td>48/F E− Sibling</td>
<td>TBI + AZQ + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 4-Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1190</td>
<td>AML-Ref</td>
<td>11/M 8/F</td>
<td>8/F E− Sibling</td>
<td>TBI + AZQ + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 1-Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1162</td>
<td>RAEB</td>
<td>34/M 32/F</td>
<td>32/F E− Sibling</td>
<td>TBI + Carboplatinum + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>Yes, Dead, 4-GF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1225</td>
<td>RAEB</td>
<td>56/F 49/M</td>
<td>49/M E− Sibling</td>
<td>TBI + Carboplatinum + Cy</td>
<td>Donor Mixed</td>
<td>None</td>
<td>DFS, 6+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1149</td>
<td>CML-CP</td>
<td>41/F 44/M</td>
<td>44/M E− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 9+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1157</td>
<td>CML-CP</td>
<td>38/M 40/M</td>
<td>40/M CD4− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 8+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1171</td>
<td>CML-CP</td>
<td>36/M 44/F</td>
<td>44/F E− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 6+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1172</td>
<td>CML-CP</td>
<td>42/M 46/F</td>
<td>46/F CD4− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>Grade 2</td>
<td>3-GVHD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1184</td>
<td>CML-CP</td>
<td>30/F 28/F</td>
<td>28/F CD4− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 6+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1185</td>
<td>CML-CP</td>
<td>47/M 28/F</td>
<td>28/F CD4+ Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>Grade 3</td>
<td>3-GVHD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1188</td>
<td>AML, 1</td>
<td>50/M 51/M</td>
<td>51/M E− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 4+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1191</td>
<td>AML, 1</td>
<td>37/M 42/F</td>
<td>42/F E− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1192</td>
<td>AML, 1</td>
<td>43/F 51/F</td>
<td>51/F E− Sibling</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1211</td>
<td>CML-AP</td>
<td>45/M 43/F</td>
<td>43/F E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>Yes, Dead, 3-Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1152</td>
<td>CML-CP</td>
<td>45/M 42/M</td>
<td>42/M E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>Yes, Dead, 3-Infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1177</td>
<td>AML-Rel</td>
<td>16/M 21/M</td>
<td>21/M E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>Yes, Dead, 5-Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1181</td>
<td>ALL, 3</td>
<td>6/M 44/F</td>
<td>44/F E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 4+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1182</td>
<td>Lymphoma</td>
<td>8/M 24/F</td>
<td>24/F E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>Grade 1</td>
<td>4+ Rel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1186</td>
<td>ALL, 3</td>
<td>8/M 41/F</td>
<td>41/F E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 4+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1189</td>
<td>CML-AP</td>
<td>36/F 43/M</td>
<td>43/M E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Donor</td>
<td>None</td>
<td>DFS, 3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1198</td>
<td>CML-CP</td>
<td>49/F 37/M</td>
<td>37/M E− Unrelated</td>
<td>TBI + Thiotepa + Cy</td>
<td>Host</td>
<td>Yes</td>
<td>Yes, Dead, 1-GF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AML, 1 or 2, first or second remission; AP, accelerated phase; CP, chronic phase; BC, blast crisis; RAEB, refractory anemia with excess blasts; Rel, relapse; Ref, refractory to induction; DFS, disease-free survivor; IP, interstitial pneumonia; Lymph, lymphoma; GF, graft failure; Grans, granulocytes; E−, sheep erythrocyte-negative cells; CD4+, cells negative for the T-cell MoAbs CD5 and CD8.
or by using a panning technique with covalently linked monoclonal antibodies (MoAbs) to CD5 and CD8.10

Isolation of genomic DNA. High molecular weight DNA was extracted from peripheral blood mononuclear cells (PBMCs) obtained from the donor and recipient before the transplant.11 After the transplant, DNA was extracted from the PB of the patient to allow determination of chimerism. In the early period post-BMT, when there was no evidence of myeloid engraftment on examination of the blood film, PBMCs were prepared for analysis. When neutrophils or monocytes were seen on the blood film, the samples were processed to allow the neutrophils and the T cells to be studied separately. In experiments performed to assess the sensitivity of the PCR technique, a single- or double-cell suspension was added directly to the PCR reaction tube as described by Jeffreys et al.8

Buffy coat cells were layered over discontinuous density gradients of 50%, 55%, 65%, and 75% Percoll and centrifuged at 40g for 30 minutes. Cells obtained from the 65% to 75% interface were greater than 99% granulocytes, as confirmed by morphologic examination of cytospin preparations. Cells removed from the two lowest density fractions were pooled and incubated at 4°C for 30 minutes in saturating concentrations of the MoAb UCHT1 (CD3).12 After washing in CaCl2-free Hank’s Balanced Salt Solution (HBSS), the cells were incubated with sheep antimouse immunomagnetic beads (Dynal, Oslo, Norway) at a beads:cell ratio of 4:1 for 30 minutes at 4°C. The CD3+ T cells were positively selected in the presence of a magnet.13 All cells isolated post-BMT received a final wash and were resuspended in HBSS at a concentration of 1.18 x 106/mL. Ten thousand (8.5 μL) PBMCs, granulocytes, or T cell/magnetic bead conjugates were added directly to PCR tubes containing a “lysis buffer” with a total volume of 20 μL containing 1 × PCR buffer [45 mmol/L Tris-HCl, pH 8.8, 11 mmol/L (NH4)2SO4, 4.5 mmol/L MgCl2, 6.7 mmol/L 2-mercaptoethanol, 4.5 μmol/L EDTA (ethylenediaminetetraacetate), and 110 μg/mL DNase-free bovine serum albumin (BSA)], 20 μmol/L dithiothreitol, 1.7 μmol/L sodium dodecyl sulfate (SDS), and 50 μg/mL proteinase K.14 To prevent evaporation, 50 μL of mineral oil was added to the PCR tubes, which were then incubated for 60 minutes at 37°C followed by 5 minutes at 95°C to inactivate the proteinase K. The samples were stored at 4°C until analysis.

PCR primers and allele-specific oligonucleotide probes for analysis of minisatellites. For PCR amplification, we synthesized specific primers designed to flank the repeat units of the following human minisatellite regions: 33.6, 33.1, MS51, α3, 3’HVR, and YNZ-22 (Table 2).8,15,16 These loci were selected for amplification for three reasons: (1) they exhibit a high degree of polymorphism; (2) many of the alleles are small and therefore amenable to PCR amplification; and (3) the alleles have been mapped to 6 different chromosomes, which is important in studies of sibling pairs in whom there is a 25% chance of inheriting an identical set of alleles on any pair of autosomes (Table 2). Allele-specific oligonucleotide probes were designed to be complementary to 1 or 2 of the tandem repeat units of the human minisatellites and used as hybridization probes. Oligonucleotide primers and probes were synthesized on an Applied Biosystems 380B DNA synthesizer (Foster City, CA). The sequences of both the PCR primers and probes are shown in Table 2.

PCR. All reactions were performed in a volume of 50 μL containing 1 × PCR buffer; 50 pmol of each primer; 1 mmol/L each of dATP, dCTP, dGTP, and dTTP; and 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). The DNA template was either prepared from 10,000 post-BMT cells as described above or from 50 to 100 ng of pre-BMT DNA extracted from the donor and recipient before transplant. Samples were overlaid with mineral oil and heated to 94°C for 7 minutes, followed by 30 cycles of amplification at the following temperature parameters: 1 minute and 20 seconds at 94°C; 1 minute at 64°C (60°C for minisatellite 33.1); and 4 minutes at 72°C. After the last cycle, the samples were held at 72°C for 10 minutes and, finally, at 4°C until analyzed.

Extensive measures were taken to minimize contamination. All samples were handled with disposable gloves, which were changed after any spillage and at frequent intervals. Preparation of blood samples, DNA extraction, and PCR preparation were performed in a laminar flow hood. All reagents and PCR reactions were prepared using pipette tips incorporating filters to prevent aerosol contamination. The thermal cycler and reaction tubes containing PCR product were kept in a separate room from where samples were handled, and separate pipettes and racks were used in the PCR-designated room. A negative control with no DNA was run with every assay. If bands were found on the gel that did not correspond to those of the donor or recipient, the results were discarded.

Analysis of PCR products. A 20-μL aliquot from each amplified reaction mixture was mixed with 2 μL 10× Ficoll loading buffer (100 mmol/L Tris-borate, pH 8.3, 2 mmol/L EDTA, 0.5% bromophenol blue, 0.5% xylene cyanol, 30% Ficoll) and subjected to electrophoresis in a 1% agarose gel containing 1 μg/mL ethidium bromide. Electrophoresis was performed in 1× TAE (40 mmol/L Tris-acetate and 1 mmol/L EDTA) for 2 hours at 100 V. The gel was then photographed by UV transillumination. The electrophoresed DNA was then transferred to Hybond (Amersham, Arlington Heights, IL) nylon membrane by capillary transfer and fixed to the membrane by UV irradiation. The membrane was prehybridized for 60 minutes at 65°C in 5× SSPE (50 mmol/L NaH2PO4, pH 7.4, 0.9 mmol/mL NaCl, and 5 mmol/L EDTA). 5× Denhardt’s solution (0.5 g Ficoll, 0.5 g polyvinylpyrrolidone, 0.5 g BSA, and H2O to 500 mL), 0.5% SDS, and 20 μg/mL salmon sperm DNA (Sigma, St Louis, MO). The membrane was subsequently hybridized for 2 hours at 65°C after the addition of the 3′-α-32P-labeled allele-specific oligonucleotide probe. The membrane was washed twice with 1× SSC (0.15 mmol/L NaCl and 0.015 mmol/L sodium citrate), 0.1% SDS for 15 minutes at room temperature, followed by a wash in 0.1× SSC, 0.1% SDS for 30 minutes at the hybridization temperature.
temperature. Membranes were then autoradiographed with two intensifier screens for 2 hours at −70°C.

**Statistics.** Analysis of significance was performed using Fisher's exact test.

**RESULTS**

*Sensitivity of the PCR method.* We first evaluated the sensitivity of the PCR by adding a known number of cells (0, 1, 2, and 100) to 20 μL of the “lysis buffer,” followed by 30 cycles of amplification. Figure 1 shows that 2 alleles can be visualized from a single cell using 32P-labeled oligonucleotide probe when the patient is heterozygous. As we added 10,000 cells directly to the “lysis buffer” for all the post-BMT assays, the sensitivity of the PCR was limited to detecting 1 in 10,000 residual host cells. Although the method appears to be semiquantitative, no attempt was made to estimate the percentage of residual host cells. By using this very sensitive technique, we were able to detect mixed chimerism in some patients without using the allele-specific probes simply by viewing the agarose gel under UV light (Fig 2).

**Kinetics of engraftment.** We studied six patients to assess the kinetics of engraftment after T-cell–depleted BMT. PB was analyzed every 2 to 3 days from day +1 to day +30 post-BMT. All studies indicated the presence of circulating donor PBMCs in the first day post-BMT. During the first 7 days post-BMT, we found both donor and host PBMCs present in the PB (Fig 3), with the host-specific allele gradually disappearing. By day +14, the granulocytes and T cells were exclusively of donor origin, but by day +25 to +30, a different pattern was emerging. The granulocytes remained of donor origin; however, the T cells were all donor in four of six patients studied, but were mixed chimeric in the other two patients (Fig 3B).

**Impact of conditioning regimen on chimerism.** We studied 29 patients who received a T-cell–depleted BMT at +26 to +30 days after transplantation. One patient (UPN 1198) who had received conditioning with TBI plus thiotepa plus Cy and marrow from an unrelated donor had no evidence of myeloid engraftment. The PB lymphocytes from this patient were of host origin. The granulocytes from the other 28 patients were of donor origin, regardless of the conditioning regimen. Of the patients who received TBI plus thiotepa plus Cy, 14 of 17 had T cells that were all donor. On the other hand, of the 12 patients who received a different conditioning regimen, only two had T cells exclusively of donor origin ($P < .001$). This effect of TBI plus thiotepa plus Cy in promoting full donor chimerism at day +28 was seen in all nine sibling transplants and in five of eight patients who received marrow from unrelated donors. Once mixed chimerism was detected, subsequent studies on the same patients between 3 and 6 months post-BMT continued to show that the granulocytes remained completely of donor origin with persistence of host T cells. Of nine patients who were complete donor chimeras at day +28, four without evidence of GVHD were found to have reappearance of residual host T cells when retested 3 to 6 months post-BMT. Therefore, in some patients, TBI plus thiotepa plus Cy only delayed the detection of residual host T cells in the PB.

**Correlation of chimeric status and clinical outcome.** Twenty-eight patients had documented evidence of myeloid engraftment within 21 days of BMT. There was no significant difference in the speed of engraftment between those patients who were complete donor chimeras and those patients who were mixed chimeras. The median times to achieving an absolute neutrophil count of $500 \times 10^9$/L and a platelet count of $50 \times 10^9$/L were 15 (range, 11 to 29) and 27 (range, 14 to 82) days, respectively, for full donor chimeras and 14 (range, 9 to 24) and 32 (range, 17 to 64) days for mixed chimeras. Although patients with mixed chimerism had rapid initial engraftment, 3 of 12 patients (UPNs 1072, 1162, and 1176) developed late graft failure 51 to 74 days post-BMT, whereas none of the 16 patients with full donor chimerism at day +28 developed graft failure.
MYELOID AND LYMPHOID CHIMERISM AFTER BMT

The results of the kinetics of engraftment studies showed that lymphoid and myeloid cells were entirely of donor origin by 2 weeks post-BMT, even in patients who were subsequently found to be mixed chimeric by 4 weeks post-BMT. Furthermore, once a patient was found to be mixed chimeric in the T-cell lineage, all subsequent studies on that patient confirmed the presence of host T cells. These findings suggest that mixed chimerism detected as early as 4 weeks post-BMT is significant when found in the PB and does not result from the persistence of dying host cells.

Donor T cells infused with the BM may be crucial for the development of complete donor chimerism. Several studies have shown that T-cell depletion of the donor marrow results in an increased incidence of mixed chimerism and graft failure when compared with conventional transplants.17-19 These studies suggest that the T cells in the marrow inoculum play a pivotal role in eliminating residual host immunocompetent lymphocytes that survive the conditioning regimen. Another factor reported to be important in determining the degree of chimerism achieved after a T-cell–depleted BMT is the pretransplant conditioning regimen. An increased incidence of mixed chimerism has been reported to be associated with fractionation of the TBI, low midline dose TBI, and the use of chemotherapy-only regimens.20-22 More immunosuppressive conditioning regimens incorporating additional chemotherapy, such as thiotepa, total lymphoid irradiation,23 or in vivo MoAb therapy,24 may facilitate engraftment. Despite these measures, a minority of patients remain at risk of graft failure. The risk of graft failure is particularly increased in patients receiving mismatched or unrelated donor transplants.

All but one of the patients in this study received identical hyperfractionated TBI plus Cy conditioning, with the only variable being the additional chemotherapy. Therefore, it is of note that thiotepa was associated with a significant increase in donor T-cell chimerism in the first 4 weeks post-BMT when compared with the other chemotherapeutic agents. Our results are in agreement with those of Terenzi et al,23 who showed that thiotepa was able to promote donor engraftment in an allogeneic mouse model that used T-cell–depleted marrow. Terenzi et al23 suggested that thiotepa promoted engraftment by its myeloablative effect. Our data suggest that the speed of myeloid engraftment was not influenced by the addition of thiotepa to the conditioning regimen, but rather that thiotepa promoted engraftment by an immunosuppressive action in either preventing or delaying the recovery of host immunocompetent T cells that are thought to be the mediators of graft rejection.

The incidence of mixed chimerism in our study was 45% at day +28, with approximately one-half of remainder of the patients becoming mixed chimeras at a follow-up of 3 to 6 months. Our data suggest that mixed chimerism may be associated with a higher incidence of graft failure, a finding that is in agreement with other published studies.18-24 It was

DISCUSSION

In this study, 29 patients were prospectively evaluated for the development of mixed myeloid and T-cell chimerism after T-cell–depleted allogeneic BMT. Although all patients studied were complete donor chimeras in their granulocyte series, 13 of 29 (45%) had evidence of residual host T cells within 4 weeks of BMT. The use of the TBI plus thiotepa plus Cy conditioning regimen resulted in more complete donor chimeras at day +28, with possibly fewer late graft failures.

Interestingly, none of the 12 patients with mixed chimerism at day +28 developed acute GVHD, whereas 5 of 16 patients with complete donor chimerism developed this complication (P = .053). A factor not associated with promoting complete donor chimerism was the use of post-BMT antithymocyte globulin as graft rejection prophylaxis. Of the 14 patients who received this treatment, 6 were complete donor chimeras and 8 were mixed chimeras.

(P = .067).
also notable that no patient with mixed chimerism developed clinical GVHD and that all five patients who developed GVHD were complete donor chimeras.

Immunologic tolerance to host immunocompetent normal cells manifested by mixed chimerism might also result in tolerance to residual leukemic cells resulting in a loss of the graft-versus-leukemia (GVL) effect. This loss of GVL activity has been reported to be a particularly severe problem after T-cell–depleted transplants for chronic myeloid leukemia (CML). Although mixed chimerism has been reported to be associated with an increased incidence of relapse, the limited follow-up and the heterogeneity of the patients’ disease status at BMT in this study do not allow us to come to any firm conclusions on relapse. However, interestingly, of four CML patients (2 chronic phase, 1 accelerated phase, and 1 blastic transformation) who have so far studied using PCR to detect BCR-ABL RNA, only the patient with mixed T-cell chimerism has evidence of molecular relapse.

Fractionation of PB or BM samples into different hematopoietic cell lineages has only occasionally been reported for the investigation of chimerism in leukemia patients. The fractionation techniques used in these studies have ranged from simple Ficoll-Hypaque density separation with E-rosetting to complicated and time consuming cell sorting. We decided to use Percoll gradients and positive selection with MoAbs and immunomagnetic beads. This had the advantage of generating relatively pure populations of granulocytes and T cells in less than 2 hours, but was also a technique that could be of value to laboratories without access to a cell sorter. Importantly, the inclusion of the immunomagnetic beads into the PCR reaction did not reduce the amount of PCR product generated. This study has shown that fractionation of the blood can result in different results in the same patient. In particular, myeloid chimerism and the speed of myeloid reconstitution was not predictive of graft failure.

Assessing chimerism using PCR to detect human minisatellites on specific cell lineages could allow other studies to be performed. Possible applications could include the detection of transfusion-induced GVHD, and chimeric status of PB natural killer (NK) and B cells and BM stromal elements post-BMT. For example, we have already documented maternal T-cell engraftment in a patient with severe combined immune deficiency (data not shown). In addition, although exquisitely sensitive leukemia-specific PCR-based methods are available for detecting minimal residual disease in acute lymphoblastic leukemia (ALL) and CML, the assessment minimal residual disease in acute myeloid leukemia (AML) is more difficult. PCR amplification of purified BM myeloid cells to determine myeloid chimerism might provide a useful tool in predicting relapse after allogeneic BMT. One possible pitfall of such a strategy would be false-positive results seen in the context of a remission marrow with residual host nonleukemic myeloid cells that survived the conditioning therapy.

In this study, we confirm that the use of a PCR-based technique allows for rapid and sensitive detection of residual host cells after T-cell–depleted BMT. With further prospective studies of different cell lineages and subsets within lineages, a better understanding of transplant biology may be possible. The use of these techniques should allow the assessment of new conditioning regimens or T-cell–depletion methods to promote complete donor chimerism.

REFERENCES


12. Burns GF, Boyd AW, Beverley PCL: Two monoclonal anti-human T lymphocyte antibodies have similar biologic effects and recognize the same cell surface antigen. J Immunol 129:1451, 1982


Myeloid and lymphoid chimerism after T-cell-depleted bone marrow transplantation: evaluation of conditioning regimens using the polymerase chain reaction to amplify human minisatellite regions of genomic DNA

S Mackinnon, L Barnett, JH Bourhis, P Black, G Heller and RJ O'Reilly