Amplification by the Polymerase Chain Reaction of Hypervariable Regions of the Human Genome for Evaluation of Chimerism After Bone Marrow Transplantation


We combined the polymerase chain reaction (PCR) with oligonucleotide hybridization as a novel and sensitive technique to evaluate posttransplant chimerism. Specific oligonucleotides for hybridization were synthesized homologous to tandemly repetitive core sequences of regions with a variable number of tandem repeats (VNTRs). Polymorphisms at such loci result from allelic differences in the number of repeats. Primers flanking the repeat region of each of the corresponding VNTRs were used for amplification. Recipient and donor pretransplant DNA and recipient posttransplant DNA were amplified. The resultant fragments were analyzed after gel electrophoresis either by hybridization in-gel or after Southern transfer. To confirm our findings, we also performed standard assays of restriction fragment length polymorphisms (RFLPs). Evaluation of 13 selected cases indicated mixed chimerism (4), complete chimerism (5), recurrence of leukemia (2), and endogenous repopulation of hematopoiesis (2) after marrow transplantation. Sensitivity of the method was determined by mixing various proportions of recipient and donor DNA; the limit of detection of the minor component in a mixture was 0.1%. PCR data correlated with RFLP data in all cases except two in which PCR proved more sensitive than RFLP. PCR amplification of VNTRs combined with oligonucleotide hybridization is a novel technique for documenting posttransplant chimerism and has advantages over RFLP analysis: high sensitivity, use of small amounts of DNA (250 ng), ease of preparation of DNA, elimination of need for restriction enzymes, and the ability to complete studies in 2 days.

R E S T R I C T I O N fragment length polymorphisms (RFLPs) are created by either loss or gain of a restriction enzyme cleavage site or by insertion or deletion of DNA between restriction sites.1,2 The former are generally two allele polymorphisms, the latter are often multiallelic if the insertions or deletions result from tandemly repeated elements. RFLPs are useful genetic markers for documentation of hematopoietic chimerism after bone marrow transplantation (BMT) as well as for determining the origin of leukemic cells in BMT patients with recurrent disease.3,4 Hypervariable regions of the human genome are highly polymorphic owing to variation in the number of tandemly repeated sequences in different alleles (so-called VNTR loci).5 These markers are inherited in a Mendelian fashion and can be very informative when a locus has multiple alleles. Generally, these multiallelic polymorphisms have been the most useful for study of marrow engraftment after allogeneic BMT.6

We previously showed that the synthetic oligonucleotide probes complementary to VNTR repeat units are capable of detecting mixed hematopoietic chimerism when the DNA of the recipient was present at 1% to 2% of the total leukocyte DNA.7,8 Engraftment of donor marrow could be determined 15 days posttransplant.9 When limited amounts of DNA are available from a patient, however, this analysis is not always possible.

Introduction of the polymerase chain reaction (PCR) as a method for the rapid amplification of DNA in vitro has provided a powerful tool for analysis of human polymorphisms.1,12 Recently, Skolnick and Wallace13 proposed that amplification of multiple polymorphisms and their simultaneous analysis provides a way to perform gene mapping or genotyping rapidly. Such amplified sequence polymorphisms (ASP) are ideal for evaluation of hematopoietic chimerism after allogeneic BMT between sibling pairs because several polymorphic loci must be analyzed to achieve a high probability of having at least one informative locus. The major advantage of using a PCR-based analysis for this purpose is increased sensitivity; ie, it is possible to detect a small proportion of DNA of donor or recipient origin. In addition, because the quality of the DNA used in the analysis is less critical, abbreviated methods may be used for DNA preparation, only small amounts of DNA are required, and the analysis can be performed more rapidly than traditional RFLP analysis. We demonstrate the utility of this method in an analysis of 13 patients who received allogeneic BMT.

MATERIALS AND METHODS

Patients. Thirteen patients who had been treated with BMT were selected to illustrate the effectiveness of our methods to analyze chimerism posttransplant. Thus, the percentage of cases of mixed chimerism, endogenous repopulation of the marrow, and complete chimerism are not representative of our entire experience. These patients were tested for marrow chimerism by PCR amplification/oligonucleotide hybridization analysis (ASP) described below, as well as by conventional RFLP analysis as previously described.10,11 Chromosome analyses were performed by standard methods when pertinent.

From the Departments of Molecular Biochemistry and Hematology and Bone Marrow Transplantation, City of Hope National Medical Center, Duarte, CA; the Department of Pathology, University of California, Los Angeles; and Laboratorio di Immunogenetica, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy.

Submitted April 16, 1990; accepted November 30, 1990.

Supported in part by Grants No. CA23175 and CA30206 from the National Cancer Institute, Bethesda, MD, and by Grant No. 9000125PF70 from the Progetto Finalizzato Biotecnologie. S.J.F. and R.B.W. are members of the Cancer Center of the City of Hope, National Institutes of Health Grant No. CA33572. L.U. was supported by a fellowship from Associazione Italiana per la Ricerca sul Cancro.

Address reprint requests to R.B. Wallace, PhD, Department of Molecular Biochemistry, Beckman Research Institute, 1450 E Duarte Rd, Duarte, CA 91010.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.

0006-4971/91/7707-00233300/0
We defined a patient as a "complete chimera" when only donor-type genetic markers could be detected from hematopoietic cells after BMT. We defined a transplant recipient as a "mixed hematopoietic chimera" or "mixed chimera" when a mixture of donor and host hematopoietic or lymphohematopoietic cells could be detected on day 14 or later after BMT. We excluded from the category of "mixed chimeras" patients who had a mixture of host and donor cells detectable only at or near the time of relapse of their leukemia, ie, when the relapse occurred within 90 days of reemergence of detectable host cells.

Isolation of genomic DNA. Blood samples or, in a few cases, BM samples were obtained from the patient and from the marrow donor before BMT and from the patient at varying intervals subsequent to the transplant. High-molecular-weight (mol wt) DNA was extracted according to the method of Kunkel et al with minor modifications.

PCR primers and locus-specific oligonucleotide probes for analysis of ASP. For PCR amplification, we synthesized specific primers designed to flank the repetitive units of the following VNTR regions: 33.1, 33.4, 33.6, H-ras, 3' HVR-globin, and YNZ-22 (Table 1). We selected these loci for amplification because known alleles were less than 2 kilobases (kb) in length. This avoids the reduced amplification efficiency reported for VNTR alleles greater than 2 kb. Sensitivity would be decreased if an informative VNTR allele greater than 2 kb were to be amplified.

Locus-specific oligonucleotides (LSO) were designed to be complementary to one or two of the respective tandem repetitive sequences and used as hybridization probes. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer (Foster City, CA) by the phosphoramidite method. They were purified by urea/polyacrylamide gel electrophoresis (PAGE) followed by high-performance liquid chromatography (HPLC) with use of a PRP-1 reverse-phase column (Hamilton). The sequences of both PCR primers and LSO are shown in Table 1.

PCR. All reactions were performed in a volume of 50 μL containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 0.01% (wt/vol) gelatin, 12.5 pmol each primer, 250 ng purified template DNA and 0.2 mmol/L each dATP, dCTP, dGTP, and TTP; 50 μL mineral oil was added to prevent evaporation. The reaction mixture was boiled 5 to 7 minutes and the mixture was allowed to cool slowly. Three to 5 U thermostable Thermus aquaticus DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) was used in each reaction. The cycle was continued for loci 33.1, 33.4, 33.6, and H-ras by annealing the primers at 64°C for 2 minutes and extending the primers at 72°C for 6 minutes. For loci 3' HVR-globin and YNZ-22, annealing and extension were performed at 72°C for 6 minutes. After extension, the samples were denatured at 95°C for 1 minute. The annealing, extension, and denaturation cycle was repeated 20 times. After the last cycle, the samples were incubated at 64°C for 2 minutes, at 72°C for 10 minutes, and finally at 4°C until analyzed.

Analysis of PCR products. An aliquot (15 μL) from each amplified reaction mixture was mixed with 3 μL 5X Ficoll loading buffer (1X = 10 mmol/L Tris-HCl, pH 7.5/1 mmol/L EDTA/0.05% bromophenol blue/0.05% xylene cyanol/3% Ficoll) and subjected to electrophoresis in a 1.5% agarose gel (Seakem, FMC, Rockland, ME). Electrophoresis was performed in 89 mmol/L Tris-HCl/89 mmol/L borate/2 mmol/L EDTA buffer for 3 hours at 120 V. The gel was subsequently stained with ethidium bromide (1 μg/mL) for 20 to 25 minutes, destained in water for 15 minutes, and photographed by ultraviolet (UV) transillumination. The ethidium bromide DNA was then transferred to Genetran (Plasco, Woburn, MA) or Sureblot (Onco, Gaithersburg, MD) nylon membrane by alkaline transfer and fixed to the membrane by UV irradiation.

The membrane was prehybridized in 5x SSPE (1x SSPE = 10 mmol/L sodium phosphate pH 7.0, 0.18 mol/L NaCl and 1 mmol/L EDTA), 1% sodium dodecyl sulfate (SDS), 10 μg/mL homomix RNA and 0.5% dehydrated powdered skim milk (Carnation, Los Angeles, CA) for 1 hour and subsequently hybridized with 0.5 to 1× 10⁶ cpm/mL at 37°C for 1 to 2 hours at 65°C. The membrane was washed twice with 6× SSC for 30 minutes at room temperature and autoradiographed with two intensifier screens (Lightning Plus; Dupont, Wilmington, DE) for 30 to 60 minutes at −70°C.

In-gel hybridization was performed with the labeled oligonucleotide in 5× SSPE, 0.1% sonicated denatured salmon-sperm DNA SDS, 10 μg/mL, and 1× 10⁶ cpm/mL labeled probe (at 65°C) for 1 hour. The gel was washed throughout with 6× SSC (1× SSC = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate) twice for 15 minutes each time at room temperature, followed by a 5-minute wash at the temperature used for hybridization with the probe.

RESULTS

Informativeness of the LSO probes. The observed heterozygosity of the six loci selected for this study ranged from 0.48 at locus 3' HVR-globin to 0.96 at locus 33.6. Table 2 shows the observed number of alleles, the most frequent allele observed, the observed heterozygosity, and the fraction of sib pairs with informative differences at the six loci used in this study. Figure 1 shows an example of the

<table>
<thead>
<tr>
<th>Locus</th>
<th>Map Location</th>
<th>Locus Symbol</th>
<th>LSO (5' to 3')</th>
<th>5' PCR Primer (5' to 3')</th>
<th>3' PCR Primer (5' to 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>33.6</td>
<td>1q</td>
<td>D1S111</td>
<td>CTCCTCGCCCTCTCTCTCCCT-AGGCCCT</td>
<td>TGTGAGTAGAGGAGAC-CTCAC</td>
<td>AAAGAACCAAGATGAGGAGG</td>
<td>7</td>
</tr>
<tr>
<td>33.1</td>
<td>ND</td>
<td>Unassigned</td>
<td>GTGCTGGTCTCCCTCTCTCTCTCTCTCAGCT</td>
<td>GTGTGACCAAGACAGCAGAGC</td>
<td>TGGTTCACCTGGCGATTGGA</td>
<td>7</td>
</tr>
<tr>
<td>33.4</td>
<td>ND</td>
<td>Unassigned</td>
<td>GGGGCCACCCACATCTG-AGGGCCACAGGGA</td>
<td>ATGGGGGAGCCGGCCACCACG</td>
<td>CAGGAGCCACACACAGA</td>
<td>7</td>
</tr>
<tr>
<td>H-ras</td>
<td>11p15.5</td>
<td>HRAS</td>
<td>GTCCTCGGCTCCCTCTCTCTCTCTTGGGCA</td>
<td>TGGGGGAAGAAGTACGCAGGGA</td>
<td>CTTCTGCAAGAGTTGACC</td>
<td>16</td>
</tr>
<tr>
<td>HVR 3'-globin</td>
<td>16p13.3</td>
<td>D16S85</td>
<td>GTGCCTGGTCTCCCTCCCTCTCTCTCTCTCCACG</td>
<td>AGTCCTCCCTCCCTCTCTCTCTCAGGA</td>
<td>GTCCATCTGGATTGTCAGTG</td>
<td>17</td>
</tr>
<tr>
<td>YNZ-22</td>
<td>17p13.3</td>
<td>D17S55</td>
<td>TTGCTGGTGGATAGGAGGAGGAGGGAAGGAGGAGGAGG</td>
<td>GTGCGGAAAAAGGAGGAGGAGGAGGAGG</td>
<td>GCCCATGATCTCTTGGAGT</td>
<td>18</td>
</tr>
</tbody>
</table>

Abbreviation: LSO, locus-specific oligonucleotide probe.

*Human Gene Mapping Library, Yale-Howard Hughes Medical Institute, New Haven, CT.
Table 2. Informativeness of Locus-Specific Oligonucleotide Probes

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. of Alleles (observed)</th>
<th>Frequency of the Most Common Allele (observed)</th>
<th>Heterozygosity (observed)</th>
<th>Fraction of Sibling Pairs Showing Informative Differences (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33.1 (n = 27)</td>
<td>10</td>
<td>0.3</td>
<td>0.79</td>
<td>82 (sp = 11)</td>
</tr>
<tr>
<td>33.4 (n = 13)</td>
<td>9</td>
<td>0.4</td>
<td>0.77</td>
<td>37 (sp = 6)</td>
</tr>
<tr>
<td>33.6 (n = 24)</td>
<td>13</td>
<td>0.2</td>
<td>0.96</td>
<td>61 (sp = 13)</td>
</tr>
<tr>
<td>H-ras (n = 24)</td>
<td>11</td>
<td>0.4</td>
<td>0.7</td>
<td>71 (sp = 7)</td>
</tr>
<tr>
<td>YNZ-22 (n = 19)</td>
<td>9</td>
<td>0.4</td>
<td>0.78</td>
<td>80 (sp = 5)</td>
</tr>
<tr>
<td>3' HVR-globin (n = 23)</td>
<td>12</td>
<td>0.25</td>
<td>0.48</td>
<td>37 (sp = 8)</td>
</tr>
</tbody>
</table>

Abbreviations: n, number of unrelated individuals tested; sp, number of sibling pairs studied.

polymorphism revealed at the locus 33.6 in 14 unrelated individuals.

**Sensitivity of the ASP procedure.** To assess the sensitivity of the ASP method, mixing experiments were performed in which donor and recipient DNAs were mixed in different proportions, keeping the total amount of DNA constant. Mixtures with decreasing proportions of recipient DNA from 10% to 0.1% were amplified, an aliquot of the product was electrophoresed, and the fragments were analyzed either by hybridization in gel or after Southern transfer to a nylon membrane. The results indicate that recipient DNA can be detected even when the concentration is only 0.1% of the total (Fig 2).

**Clinical utility.** Our strategy to study the 13 donor/recipient sibling pairs by the ASP method consisted first of analyzing both patient and donor pretransplant samples with primers and probes corresponding to the three most polymorphic loci (33.1, 33.6, and H-ras). In cases in which these loci were not informative, the samples were also studied with 33.4, YNZ-22, and 3'HVR-globin. All patients had recipient-specific and donor-specific fragments, and we were able to document the presence of complete chimerism, mixed chimerism, and endogenous marrow repopulation. By these analyses, 4 of the patients had mixed chimerism, 5 had complete chimerism, 2 had recurrence of their leukemia, and 2 had endogenous repopulation of the marrow. Pertinent data regarding these patients are shown in Table 3.

**Complete chimerism.** Figure 3 shows recipient (UPN 489C) and donor ASP patterns for two different loci, H-ras and 33.6 before transplantation for acute lymphocytic leukemia (ALL). Subsequent lanes show the patient's

![Fig 1. Analysis of 14 unrelated individuals by the ASP method. Each lane contains the amplified DNA products using primers for locus 33.6. The amplified DNA was subjected to electrophoresis on an agarose gel and transferred to a membrane after in situ denaturation. The membrane was hybridized with LSO 33.6.](image)

![Fig 2. Sensitivity of the ASP approach. DNA from donor (D) and recipient (R) was mixed in the following proportions: 10%, 1%, 0.5%, 0.1% R. An aliquot of the amplified product was electrophoresed, alkaline transferred onto nylon membrane (GeneTran, Plasco), and hybridized with LSO probe 33.6. After 2-hour exposure using two intensifier screens (Dupont Lightning Plus) at -70°C, 0.1% recipient DNA was detected.](image)
Table 3. Clinical and Laboratory Findings

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>Days Post-BMT of Follow-up Spec*</th>
<th>Chimera Status</th>
<th>Clinical Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RFLP</td>
<td>ASP*</td>
</tr>
<tr>
<td>437C</td>
<td>SAA</td>
<td>20-100 (3)</td>
<td>CC</td>
<td>MC (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 (BM)</td>
<td>MC (R)</td>
<td>MC (R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>230</td>
<td>ER</td>
<td>ER</td>
</tr>
<tr>
<td>451C</td>
<td>AML</td>
<td>20-60 (3)</td>
<td>MC (&gt; 95% D)</td>
<td>MC (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+20</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td>520C</td>
<td>ALL</td>
<td>20-45 (3)</td>
<td>CC</td>
<td>MC (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21 (BM)</td>
<td>CC</td>
<td>MC (D)</td>
</tr>
<tr>
<td>605U</td>
<td>SAA</td>
<td>+30-37 (3)</td>
<td>ER</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td></td>
<td>88</td>
<td>ER</td>
<td>ER</td>
</tr>
<tr>
<td>615U</td>
<td>SAA</td>
<td>33</td>
<td>MC (&gt; 90% D)</td>
<td>MC (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>MC (&gt; 90% D)</td>
<td>MC (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94</td>
<td>MC (&gt; 90% D)</td>
<td>MC (D)</td>
</tr>
<tr>
<td>642U</td>
<td>CML</td>
<td>15</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32-53 (2)</td>
<td>CC</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>74</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164-270 (4)</td>
<td>MC (&gt; 90% D)</td>
<td>MC (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>330-360 (2)</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td>645U</td>
<td>ALL</td>
<td>20-180 (4)</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td>648U</td>
<td>ALL</td>
<td>22-27 (2)</td>
<td>MC (90% D)</td>
<td>MC (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60-120 (3)</td>
<td>MC (50% D)</td>
<td>MC (D = R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>61 (BM)</td>
<td>MC (50% D)</td>
<td>MC (D = R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>170</td>
<td>MC (95% R, REL)</td>
<td>MC (R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>205-240 (3)</td>
<td>MC (95% R, REL)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 (2ND BMT)</td>
<td>CC</td>
<td>NT</td>
</tr>
<tr>
<td>658U</td>
<td>AML</td>
<td>29-69 (3)</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td>677U</td>
<td>AML</td>
<td>12</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td>679U</td>
<td>AML</td>
<td>25-60 (3)</td>
<td>CC</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>CC</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>MC (90% D, REL)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90</td>
<td>MC (90% D, REL)</td>
<td>MC (D, REL)</td>
</tr>
<tr>
<td>673U</td>
<td>ALL</td>
<td>29</td>
<td>CC</td>
<td>CC</td>
</tr>
</tbody>
</table>

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BM, bone marrow; C, City of Hope; CC, complete chimera; CML, chronic myelogenous leukemia; D, donor; ER, endogenous repopulation (all DNA of recipient origin); MC, mixed chimera; R, recipient; REL, relapse, hematologic; SAA, severe aplastic anemia; U, UCLA; UPN, unique patient number; NT, not tested.

*All DNA was derived from peripheral blood except when indicated as being from BM.
†Using RFLP, semi-quantitative analysis of the percentage of donor vs. recipient DNA as mixed chimeras was performed by using standards consisting of various mixtures of donor and recipient DNA.
‡Using ASPs, quantitative analyses were not performed; the predominant DNA was judged by the intensity of bands and is shown in parentheses.

posttransplant DNA pattern for DNA obtained on day +20. All DNA detected was of donor origin.

Mixed chimerism. Figure 4 shows recipient (UPN 520C) and donor phenotypes before transplantation for ALL. On days +21 (BM), +22 (peripheral blood, PB) and +45, both donor and recipient DNA were detected by ASP analysis, whereas on day +60 only donor DNA was detected, thus documenting transient mixed chimerism. In contrast, only donor DNA was detected by RFLP during this period. BM aspirations on days +30 and +60 showed marked hypocellularity but no evidence of leukemia. The patient died of cytomegalovirus (CMV) pneumonia and severe graft-versus-host disease (GVHD).

Endogenous repopulation of the marrow. Figure 5 shows recipient (UPN 605U) and donor phenotypes before transplantation for aplastic anemia. This was the patient's second transplant using the same donor because the first BMT failed to engraft. RFLP and ASP analyses made from day +30 to +88 indicated that all DNA detected was of recipient origin. BM examination after the second BMT showed marked hypocellularity with no evidence of engraftment; chromosome studies showed no dividing cells. The patient died on day +180 of graft failure.

Patient UPN 437C was transplanted for aplastic anemia. Analysis by RFLP showed only donor DNA on specimens obtained on days +20 and +45; however, ASP analysis showed a minor proportion (~5%) of recipient DNA on these specimens. On day +100, DNA extracted from PB nucleated cells and analyzed by ASP and RFLP was predominantly of donor origin whereas DNA from BM cells was predominantly of recipient origin. On day +230, only the recipient pattern was evident. The patient is in remission with the following hematologic values: hemoglobin 12.5 g/dL, WBC 6,600/μL, and platelet count 291,000/μL (manuscript in preparation).

Recurrent leukemia. Patient UPN 648U was trans-
PCR ANALYSIS OF BMT CHIMERISM

Fig 3. Complete chimerism. DNA from patient UPN 489C was analyzed using primers and LSO specific for loci H-ras and O-33.6. R and D represent the pretransplant recipient and donor PB DNAs. A recipient-specific fragment is evident before transplantation, but only DNA from the donor is present on day +20 after transplantation.

planted for ALL. As shown in Fig 6, mixed chimerism was detected by ASP analysis on the first evaluation after BMT on day +22 and there was an increase in the relative concentration of the recipient-specific band on days +60, +63, and +82. On day +170, the DNA was predominantly of recipient origin. The ASP and RFLP results were concordant in all specimens. The patient remained in hematologic remission until day +165, at which time BM examination indicated relapse of his leukemia. The patient was transplanted a second time using the same donor, and on day +20 after the second BMT, RFLP analysis showed complete chimerism. On day +36 after second transplant, however, the patient died of liver failure attributed to toxicity of the pretransplant regimen.

Minimal residual leukemia. Patient UPN 642U was transplanted for CML. The patient had been in complete hematologic remission since transplantation; however, the various methods used to detect posttransplant residual recipient DNA have shown a lack of concordance as follows. Posttransplant DNA analysis by RFLP and ASP had indicated complete chimerism on days +15, +32, +53, and +74. On days +164, +208, +256 and +270, however, about 10% of DNA was of recipient origin. Further samples obtained at +11 months and +12 months indicated complete chimerism. Figure 7 shows the ASP analysis for days +74, +208, and +330, as well as of a sample composed of a mixture of recipient and donor DNAs in a ratio of 1:99. Amplification of the bcr rearrangement by PCR yielded positive results on a pretransplant specimen and throughout the posttransplant period on days +20, +165, +208, +270, and +330. Positive results were obtained only when primers and the corresponding LSO to the ALL junction were used. This indicates that the breakpoint occurred in the first bcr intron. Chromosome analyses showed a Philadelphia chromosome before transplantation and after transplant at +240 days, but at 1 year posttransplant, the Philadelphia chromosome was no longer detectable.

DISCUSSION

To evaluate posttransplant chimerism, we used synthetic oligonucleotide probes which, under appropriately stringent conditions of hybridization and washing, will only hybridize to produce a perfectly matched duplex between

\[
\begin{array}{c|c|c}
R & +22 & +45 +60 BM+21 \\
D & & \\
\end{array}
\]

Fig 4. Transient mixed hematopoietic chimerism. DNA from patient UPN 520C was analyzed using primers and the corresponding LSO probes specific for locus H-ras. R and D indicate pretransplant recipient and donor PB DNAs. On days +21 and +45 after transplantation, the recipient-specific fragments (1,600 bp) were present together with an allele shared by the recipient and donor. On day +60, no recipient fragments were detectable, indicating complete engraftment. BM + 21 is DNA extracted from BM on day +21 after transplantation.
and (dG-dT)n blocks have also been amplified. In this case, however, production of small fragments makes use of polyacrylamide gels necessary to resolve them. Hence, the presence of nonspecific fragments makes interpretation of the results in BMT cases difficult.\(^\text{26,29}\)

The amplification of another two hypervariable regions has been published: The apolipoprotein B 3' hypervariable region\(^\text{30}\) and the interleukin-6 3' hypervariable region.\(^\text{31}\)

Both were successfully amplified and detected using synthetic oligonucleotides; however, the observed fragment sizes ranged between 500 and 900 bp. In this instance, the fragments can be too close together to produce a clear result in BMT, especially when mixed chimeras are analyzed.

The highly polymorphic segment detected by probe pYNZ22 (HGM 10 locus D17S5) has also been amplified by PCR.\(^\text{32}\) The study of random individuals showed a high heterozygosity, and the alleles are distributed in a broad range of mol wt.

With synthetic LSO, we have been able to amplify and detect six different hypervariable regions of the human genome: 33.1, 33.4, 33.6, YNZ22, H-ras, and 3' HVR-globin, four of which have been previously amplified by PCR (described above). In every case, we obtained a clear pattern without the presence of false or nonspecific fragments.

Our results indicate that this technique for studying posttransplant chimerism has several advantages over previ-
PCR ANALYSIS OF BMT CHIMERISM

Our data also indicate a high degree of informativeness using the set of six PCR primer pairs and LSO probes. We were able to demonstrate both donor-specific and recipient-specific fragments in all 13 donor-recipient sibling pairs. These loci therefore may also be of use in paternity determination or forensic analysis. Furthermore, because of the high degree of informativeness of 33.6 primer/probe combination (Fig 1), this single primer/probe alone should be useful for analysis of most BMT involving HLA matched/unrelated donors.

We have not attempted to quantitate our results using the ASP technique. An estimate of the level of recipient DNA present in the posttransplant DNA sample can be made by comparison with appropriately mixed donor and pretransplant recipient samples. Unlike use of clonal markers such as bcr-abl, T-cell receptor delta genes, the ASP method will detect the presence of any recipient DNA, regardless of the lineage of the cell containing this DNA. This analysis must be performed in the presence of an excess of donor DNA, however, thus limiting the ultimate sensitivity of the method.

We compared the results obtained using the ASP method with previously reported methods using standard analysis of RFLP. The results correlated except in two cases in which the ASP method showed a higher degree of sensitivity. Analysis of RFLP using the synthetic probes O-AY-29 and O-YNH-24 in patient UPN 520C indicated complete chimerism, whereas mixed chimerism was detectable using ASPs on days +20, +21, +22, and +45. Similarly, in patient UPN 437C, analysis using RFLP indicated complete chimerism whereas PCR analysis showed the presence of mixed chimerism on days +20, +45, and +100 (PB) before development of endogenous repopulation of the marrow on day +230.

An alternative method of detecting residual host hematopoietic cells posttransplant is amplification of a known clonal marker of malignancy, as represented by amplification of the bcr in patients who have this rearrangement before BMT. PCR of bcr has the advantage of specific detection of the malignant clone and, at least in some instances, may be a more sensitive method of detecting small numbers of residual host cells, as illustrated by our patient UPN 642U. This difference in sensitivity is presumably the result of differences in efficiency of amplification or of sensitivity of hybridization when various probes are used. The principle of amplification of a clonal marker is applicable only in disorders in which such a marker is identifiable, however.

Because successful BMT depends on engraftment of pluripotent hematopoietic stem cells in the marrow environment and because PCR has the ability to amplify specific

The sensitivity of the ASP method for detecting mixed chimerism is high, owing mainly to the inherent sensitivity of the method of amplification using PCR. By mixing DNA from recipient and donor in different proportions, we showed a 0.1% level of detection after Southern transfer or when using in-gel hybridization. This is significantly more sensitive than conventional methods of RFLP analysis which generally have a lower limit of sensitivity of 1% to 5%. The DNA used does not require digestion by restriction fragments, which saves considerable time and expense and eliminates the problem of partial digestions of DNA. In addition, DNA may be prepared by abbreviated methods that require as little time as 1 hour because PCR amplification does not require pure DNA. In addition, the procedure requires use of only small amounts of DNA (~250 ng), which is important when the amount of DNA that can be obtained from the patient's sample is limited, eg, in the period shortly after BMT (10 to 15 days) or in infants or leukopenic patients. Only 2 days are required to obtain the final result after a specimen of PB or BM is received in the laboratory. A recently published method for rapid preparation of DNA suitable for PCR should make analysis time even shorter.

Our data also indicate a high degree of informativeness using the set of six PCR primer pairs and LSO probes. We were able to demonstrate both donor-specific and recipient-specific fragments in all 13 donor-recipient sibling pairs. These loci therefore may also be of use in paternity determination or forensic analysis. Furthermore, because of the high degree of informativeness of 33.6 primer/probe combination (Fig 1), this single primer/probe alone should be useful for analysis of most BMT involving HLA matched/unrelated donors.

We have not attempted to quantitate our results using the ASP technique. An estimate of the level of recipient DNA present in the posttransplant DNA sample can be made by comparison with appropriately mixed donor and pretransplant recipient samples. Unlike use of clonal markers such as bcr-abl, T-cell receptor delta genes, and T-cell receptor γ genes, the ASP method will detect the presence of any recipient DNA, regardless of the lineage of the cell containing this DNA. This analysis must be performed in the presence of an excess of donor DNA, however, thus limiting the ultimate sensitivity of the method.

We compared the results obtained using the ASP method with previously reported methods using standard analysis of RFLP. The results correlated except in two cases in which the ASP method showed a higher degree of sensitivity. Analysis of RFLP using the synthetic probes O-AY-29 and O-YNH-24 in patient UPN 520C indicated complete chimerism, whereas mixed chimerism was detectable using ASPs on days +20, +21, +22, and +45. Similarly, in patient UPN 437C, analysis using RFLP indicated complete chimerism whereas PCR analysis showed the presence of mixed chimerism on days +20, +45, and +100 (PB) before development of endogenous repopulation of the marrow on day +230.

An alternative method of detecting residual host hematopoietic cells posttransplant is amplification of a known clonal marker of malignancy, as represented by amplification of the bcr in patients who have this rearrangement before BMT. PCR of bcr has the advantage of specific detection of the malignant clone and, at least in some instances, may be a more sensitive method of detecting small numbers of residual host cells, as illustrated by our patient UPN 642U. This difference in sensitivity is presumably the result of differences in efficiency of amplification or of sensitivity of hybridization when various probes are used. The principle of amplification of a clonal marker is applicable only in disorders in which such a marker is identifiable, however.

Because successful BMT depends on engraftment of pluripotent hematopoietic stem cells in the marrow environment and because PCR has the ability to amplify specific

Fig 7. Minimal residual leukemia. Patient UPN 642U was transplanted for CML. The patient had been in complete hematologic remission since transplantation. Transient mixed chimerism was demonstrated by RFLP and ASP analysis (~10% of the DNA is of recipient origin) and by chromosome analysis (not shown). Amplification of the bcr by PCR has been positive throughout the posttransplant period (not shown).
DNA segments from as little as one molecule of DNA, PCR amplification and analysis of polymorphic loci could be used to determine the genotype (ie, individual origin) of cells obtained from in vitro colony forming progenitor cells. We believe that the APS method represents a significant improvement over previous methods for analysis of post-transplant chimerism as far as sensitivity, ease, minimal time requirement, and reliability is concerned.

REFERENCES

34. Gabert J, Thuret I, Lafage M, Carcassonne Y, Maraninchi
PCR ANALYSIS OF BMT CHIMERISM


Amplification by the polymerase chain reaction of hypervariable regions of the human genome for evaluation of chimerism after bone marrow transplantation

L Ugozzoli, P Yam, LD Petz, GB Ferrara, RE Champlin, SJ Forman, D Koyal and RB Wallace