Determining both lymphoid chimerism and the presence of minimal residual disease after allogeneic bone marrow transplantation (BMT) for chronic myelogenous leukemia (CML) could be helpful to the understanding of the biology of leukemic relapse in this disease. We prospectively investigated 32 patients with CML post-BMT by assessing T-cell chimerism and minimal residual disease using sensitive polymerase chain reaction (PCR) methodologies. Patients were studied between 1 and 24 months post-BMT. Thirty patients received a T-cell-depleted marrow graft and received unmanipulated marrow. All but 1 patient were conditioned with total body irradiation (TBI) + thiotepa + cyclophosphamide (Cy). The other patient received TBI + Cy as conditioning. The T cells were exclusively of donor origin in 12 of 16 patients who were tested at 1 month post-BMT, but were mixed chimeric in 11 of these patients by ≥3 months. Once mixed T-cell chimerism was documented, no patient returned to having all donor T-cells. At a median follow-up of 12 months, minimal residual disease was present in 18 of 22 patients with mixed T-cell chimerism and in 3 of 10 patients with full donor chimerism. The actuarial molecular relapse rate at 24 months for the two groups is 91% and 33%, respectively (P < .02). The finding of BCR-ABL mRNA within the first 6 months of transplant or on two consecutive assays was highly predictive of subsequent cytogenetic or hematologic relapse (P = .032 and P < .02, respectively). Ten patients, 9 with mixed T-cell chimerism, have relapsed (4 clinical, 6 cytogenetic) at a median of 12 months post-BMT. These data suggest that mixed T-cell chimerism may be a marker for abrogation of graft-versus-leukemia activity that is thought to be pivotal in eradicating minimal residual disease after BMT for CML.

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BONE MARROW transplantation (BMT) is the treatment of choice for patients with chronic myelogenous leukemia (CML) who have a suitable BM donor. Patients transplanted in chronic phase using unmanipulated donor marrow have a relapse rate of between 9% and 24%.1,2 A higher incidence of relapse and a lower incidence of graft-versus-host disease (GVHD) has been reported for T-cell-depleted grafts as compared with conventional BMT.3 Furthermore, it is well documented that recipients of T-cell-depleted BMT have a higher incidence of mixed chimerism than those patients receiving conventional transplants.4 The relationship between mixed chimerism and leukemia relapse has been a matter of controversy.4,11,12 Although mixed chimerism is not necessarily associated with a poor prognosis, several groups have suggested that mixed chimerism is associated with an increased risk of leukemia relapse.4,10,11 However, none of these studies has addressed the issue of lineage specific chimerism in a single disease. We decided to study T-cell chimerism after BMT for CML as it has been shown that the graft-versus-leukemia (GVL) effect is particularly important in eradicating residual leukemia in this disease, an activity that may be mediated by donor T cells.11 Additionally, as the T cell is not part of the leukemic clone in CML, the persistence of host T cells after transplant is a variable that is independent of detecting residual leukemia.

We describe the results of 32 patients transplanted for CML using a previously reported minisatellite polymerase chain reaction (PCR) method to determine T-cell chimerism together with a BCR-ABL mRNA PCR to detect minimal residual disease. Our results indicate that mixed T-cell chimerism is associated with not only an increased incidence of minimal residual disease, but also more cytogenetic and hematologic relapses.

**MATERIALS AND METHODS**

**Patients.** Thirty-two patients with chronic myelogenous leukemia undergoing allogeneic BMT were studied. Details of disease stage, donor type, GVHD prophylaxis, and conditioning regimen are shown in Table 1. Thirty patients received a T-cell-depleted marrow graft and two received unmanipulated marrow. All but one of the patients were conditioned with fractionated total body irradiation (TBI) 12 × 125 cGy + thiotepa 10 mg/kg + cyclophosphamide (Cy) 120 mg/kg. The other patient received TBI + Cy as conditioning. In vitro T-cell depletion was performed using soybean agglutinin (SBA) to produce a SBA-E− marrow fraction. This was further T-cell-depleted with either rosetting with sheep red blood cells to give a SBA-E− marrow or by using a panning technique with covalently linked monoclonal antibodies (MoAbs) to CD5 and CD6.5 Thirty-one donor-recipient pairs were matched at the human leucocyte antigen (HLA) A, B, and DR loci, one donor-recipient pair were HLA A and B identical, but were mismatched for one DR locus. Clinical protocols were approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center (New York, NY) and transplants were performed with the informed consent of the patients. Cytogenetic analysis was performed at three monthly intervals post-BMT. Twenty metaphases were examined on each occasion. The finding of a single Ph+ metaphase at any time was defined as cytogenetic relapse.

**Isolation of genomic DNA.** High molecular-weight DNA was extracted from peripheral blood mononuclear cells obtained from the donor and recipient before the transplant.16 After the transplant, DNA was extracted from the peripheral blood of the patient to allow determination of chimerism. Buffy coat cells were layered over discontinuous density gradients of 50%, 55%, 65%, and 75% Percoll (Pharmacia Biotech Inc, Piscataway, NJ) and centrifuged at 5000 g for 10 minutes at 4°C. DNA was extracted from 20 × 106 cells and stored in water.

**Patients.** Thirty-two patients with chronic myelogenous leukemia undergoing allogeneic BMT were studied. Details of disease stage, donor type, GVHD prophylaxis, and conditioning regimen are shown in Table 1. Thirty patients received a T-cell-depleted marrow graft and two received unmanipulated marrow. All but one of the patients were conditioned with fractionated total body irradiation (TBI) 12 × 125 cGy + thiotepa 10 mg/kg + cyclophosphamide (Cy) 120 mg/kg. The other patient received TBI + Cy as conditioning. In vitro T-cell depletion was performed using soybean agglutinin (SBA) to produce a SBA-E− marrow fraction. This was further T-cell-depleted with either rosetting with sheep red blood cells to give a SBA-E− marrow or by using a panning technique with covalently linked monoclonal antibodies (MoAbs) to CD5 and CD6.5 Thirty-one donor-recipient pairs were matched at the human leucocyte antigen (HLA) A, B, and DR loci, one donor-recipient pair were HLA A and B identical, but were mismatched for one DR locus. Clinical protocols were approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center (New York, NY) and transplants were performed with the informed consent of the patients. Cytogenetic analysis was performed at three monthly intervals post-BMT. Twenty metaphases were examined on each occasion. The finding of a single Ph+ metaphase at any time was defined as cytogenetic relapse.
Table 1. Clinical Parameters of the 32 Patients

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<th>UPN</th>
<th>Stage</th>
<th>Prophylaxis</th>
<th>Donor Type</th>
<th>Conditioning Regimen</th>
<th>Day +2B</th>
<th>= 3</th>
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Abbreviations: CP1/CP2, first/second chronic phase; AP, accelerated phase; CSA, cyclosporin A; Pred, prednisone; E−, sheep erythrocyte negative cells; CD 5/8−, cells negative for the T-cell monoclonal antibodies CD5 and CD8; SBA−, marrow T-cell depleted with soy bean agglutnin only; TBI, total body irradiation; Thio, thiota; Cy, cyclophosphamide; CR, complete hematologic and cytogenetic remission; Cy Rel, cytogenetic relapse; Hem Rel, hematologic relapse.
400g for 30 minutes. Cells obtained from the 65% to 75% interface were greater than 99% granulocytes, confirmed by morphologic examination of cytopsin 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). After washing in Ca++, Mg++-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. All cells isolated post-BMT received a final wash and were resuspended in HBSS at a concentration of 1.18 × 10^6/mL. Ten thousand (8.5 ul) 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 mM Tris-HCI pH 8.8, 11 mM (NH4)2SO4, 4.5 mM MgCl2, 6.7 mM 2-mercaptoethanol, 4.5 μM/μL EDTA and 110 μM DNAse-free bovine serum albumin (BSA)), 20 mM dithiothreitol, 1.7 μM sodium dodecyl sulfate (SDS) and 50 μg/μL proteinase K. To prevent evaporation, 50 μL of mineral oil was added to the PCR tubes that 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, X5S2, 3′HVR, APO-B, and YNZ-22.29-31 These loci were selected for amplification for three main 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 seven different chromosomes, a factor that is important in studies of sibling pairs where there is a 25% chance of inheriting an identical set of alleles on any pair of autosomes. 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).

Minisatellite PCR. All reactions were performed in a volume of 50 μL containing 1 × PCR buffer, 50 pM of each primer, 1 mM/L each of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxyctydine triphosphate (dCTP), 2.5 U Taq polymerase (Perkin Elmer, Cetus, 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 20 seconds at 94°C; 1 minute at 64°C (60°C for 33.1); 4 minutes 72°C. After the last cycle, the samples were held at 72°C for 10 minutes and finally at 4°C until analyzed.

Analysis of minisatellite PCR products. A 20-μL aliquot from each amplified reaction mixture was mixed with 2 μL 10 × Ficoll 400 (Sigma, St Louis, MO) loading buffer (100 μM/L TRIS-borate pH 8.3, 2 mM/L EDTA, 0.5% bromophenol blue, 0.5% xylene cyanol, 30% Ficoll Hypeaque) and subjected to electrophoresis in 1% agarose gel containing 1 μg/mL/mL ethidium bromide. Electrophoresis was performed in 1 × TAE (40 mM TRIS-acetate, 1 mM/L EDTA) for 2 hours at 100 V. The gel was then photographed by ultraviolet (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 mM/L NaH2PO4 pH 7.4, 0.9 mM/L NaCl and 5 mM/L EDTA), 5 × Dernhard’s solution (0.5 g Ficoll Hypeaque, 0.5 g polyvinylpyrrolidone, 0.5 g BSA and H2O to 500 mL), 0.5% SDS and 20 μg/mL salmon sperm DNA (Sigma Chemical Co, 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 probe was washed twice with 1 × SSC (0.15 M/mL NaCl, 0.015 M/mL sodium citrate), 0.1% SDS for 15 minutes at room temperature followed by a wash in 6 × SSC, 0.1% SDS for 30 minutes at the hybridization temperature. Membranes were then autoradiographed with two intensifier screens for 2 hours at -70°C.

The sensitivity of detecting mixed chimerism with this technique is between 0.1% and 1% depending on the primers used and the length of the allele being amplified. No attempt was made to quantitate mixed chimerism.

Detection of minimal residual disease. RNA was prepared from peripheral blood and BMuffy coat cells. RNA was extracted by the acid guanidinium/phenol/chloroform method. To prepare the cDNA, between 2 to 5 μg of total RNA was added to a total volume of 20 μL reverse transcriptase reaction containing 1 × reverse transcriptase buffer (50 μM/L TRIS-HCl pH 8.3, 75 mM/L KCl, 3 mM MgCl2) (GIBCO-BRL, Gaithersburg, MD), 10 mM dithiothreitol (DTT), 0.5 mM/L each of dATP, dCTP, dGTP, and dTTP, RNase inhibitor 1 μL/μL (Boehringer, Mannheim, Germany), reverse transcriptase 2.5 U/μL (GIBCO-BRL) and 2.5 μM/L random hexamers (Boehringer). Reactions were allowed to incubate at room temperature for 10 minutes followed by 60 minutes at 37°C, and 10 minutes at 94°C to inactivate the reverse transcriptase. The cDNA was then stored at 4°C until the PCR was performed.

A nested PCR technique was used as previously described. Briefly, the cDNA was then divided into 2 tubes each containing a final volume of 100 μL with 1 × PCR buffer (50 mM/L KCl, 10 mM/L TRIS-HCl pH 8.3, 1.5 mM/L MgCl2, 0.001% gelatin) (Perkin Elmer-Cetus), 0.2 mM/L each of dATP, dCTP, dGTP, and dTTP, 2.5 U Taq polymerase and 1 μg of each of the upstream and downstream oligonucleotide primers. To one half of the cDNA an ABL sense primer was added to the ABL antisense primer which results in a positive 180-bp band from all human RNA, and therefore, will control for the quality of RNA and successful PCR amplification especially in the absence of any detectable BCR/ABL fusion product amplification. To the other half of the cDNA mix, a BCR sense primer is added to the ABL antisense primer that will amplify the cDNA derived from the B2A2 (356 bp) and B3A2 (433 bp) transcripts if present. Of this PCR product, 2.5% is added to a second PCR that uses the nested ABL and BCR primers for the BCR-ABL transcript. These primers specifically amplify an internal segment of the B2A2 or the B3A2 PCR products derived from the first PCR. A programmable thermal cycling machine is used for the PCR. The block is set for 35 cycles, the first cycle being 10 minutes at 94°C, 5 minutes at 60°C, and 2 minutes at 72°C, with the remaining cycles being 45 seconds at 94°C, 45 seconds at 60°C, and 60 seconds at 72°C, and the last step being held at 72°C for 10 minutes. PCR product was run on an ethidium bromide-stained 2% agarose gel and visualized under UV light.

Using this approach we can detect a single control-positive (K562) cell in 10^6 normal cells. BCR/ABL negative cells are included in all RNA extraction procedures as negative controls to assess cross-contamination between RNA samples. A blank control is added at the cDNA stage that included all reagents except RNA to control for contamination with PCR product in any of the buffers, enzymes or primers, or cross-contamination between tubes. A dilute positive control (RNA prepared in a separate procedure from a cell preparation containing one leukemic cell per 10^6 normal cells) is added at the cDNA stage. Elaborate measures are taken to minimize contamination. The recommendations of Kwok and Higushi have been
adopted. In addition, all PCR product is kept in a separate laboratory from the patient samples, RNA, and PCR reagents.

Quantitative PCR. Quantitative PCR was performed by using serial dilution of a competitive RNA template as previously described. Briefly, we produced two separate competitive RNA templates from the K562 (B3A2) and BV173 (B2A2) CML cell lines respectively. RNA prepared from the cell lines was reverse transcribed to produce a cDNA as described above, and amplified in a single-step PCR reaction using the ABL antisense primer together with a modified BCR sense primer that has an additional 23 bases at its 5’ end incorporating the T7 RNA polymerase promoter. The double-stranded DNA PCR product was purified using chromaspin 100 columns (Clontech, Palo Alto, CA) and used as a template in an in vitro transcription reaction. The reaction included: 20 µL of 5 × transcription buffer (GIBCO, Grand Island, NY); 500 U T7 RNA polymerase (GIBCO); 5 mmol/L DTT; 0.4 mmol/L each of ATP, CTP, GTP and uridine triphosphate (Boehringer Mannheim, Indianapolis, IN); 100 U RNase inhibitor (Boehringer Mannheim); 10 µL DNA template and water to a total volume of 100 µL. After incubation at 37°C for 2 hours we removed the DNA template by adding 20 U RNase-free DNase (Boehringer Mannheim) for 1 hour at 37°C. The DNase was inactivated at 65°C for 20 minutes. The RNA was purified by phenol/chloroform extraction and quantitated. Serial dilutions of the RNA competitive templates were then prepared for use in quantitative PCR reactions. The efficiency of the DNase step was checked by amplification of 10^7 molecules of the competitive RNA templates through two rounds of PCR, each of 35 cycles with the use of nested primers for the second PCR step. In the absence of reverse transcriptase we failed to generate a detectable PCR product on agarose gels. Serial dilutions of the B2A2 RNA templates were used to compete with RNA from patients with the B2A2 rearrangement. Similarly the B3A2 templates were used to compete with RNA from patients with the B2A2 rearrangement.

For quantitative PCR analysis a mastermix containing (in the final reaction volume of 20 µL) 1 × reverse transcriptase buffer, 0.5 mmol/L each of dATP, dCTP, dGTP and dTTP, 10 mmol/L DTT, RNase inhibitor 1 U/µL, reverse transcriptase 2.5 U/µL and 2.5 µmol/L random hexamers together with 1 µg per assay of the RNA sample to be tested. Fifteen microliters of the mastermix was added to 5 µL of different dilutions of the competitive RNA template and overlaid with 70 µL mineral oil. There were four dilutions of the competitive RNA template for each log dilution, eg, 100,000; 75,000; 50,000; 25,000; and 10,000. Reactions are allowed to incubate at room temperature for 10 minutes followed by 60 minutes at 37°C, 10 minutes at 94°C to inactivate the reverse transcriptase. The cDNA was then amplified in a two-step nested PCR as described above and the PCR product subjected to agarose gel electrophoresis.

Competitive PCR was performed on each sample on two occasions. For a result to be considered valid the equilibrium point be-
Some patients who had BCR-ABL mRNA detectable at 6 months or greater post-BMT had granulocytes that were mixed chimeric. This may represent a less-sensitive PCR method for detecting residual disease.

**Mineral residual disease.** The BCR-ABL transcript was detected on at least one occasion in 23 of the 32 patients after BMT of whom 21 had detectable residual disease at last follow-up (Fig 2, A and B). Ten patients have subsequently developed either cytogenetic or hematologic relapse.

Sixteen patients have had two consecutive positive assays for BCR-ABL mRNA. Eight of these patients have gone on to cytogenetic or hematologic relapse. The finding of two consecutive positive assays for BCR-ABL was associated with an increased incidence of cytogenetic or hematologic relapse ($P < 0.02$).

Of the 23 patients who were studied in the first 6 months post-BMT, 15 were found to be PCR positive on at least one assay. None of the 8 patients who were PCR negative and 7 of the 15 patients who were PCR positive have subsequently progressed to cytogenetic or hematologic relapse at a median follow-up of only 12 months post-BMT (Fig 2, A and B). The finding of a positive assay for BCR-ABL mRNA within 6 months of transplant was associated with an increased risk of cytogenetic or hematologic relapse ($P = 0.032$).

Only 3 patients have converted from being BCR-ABL mRNA positive to negative. One of these 3 patients had received a conventional BMT, and quantitative PCR had shown an increase in BCR-ABL mRNA between 3 and 6 months post-BMT (Fig 3). The cyclosporine A and prednisone used as GVHD prophylaxis were discontinued at 6 months. Thereafter, the patient developed chronic GVHD and became PCR negative (Fig 3).

**Chimerism and residual disease.** At a median follow-up of 12 months minimal residual disease was present at last follow-up in 18 of 22 patients with mixed T-cell chimerism and in 3 of 10 patients with full donor chimerism. The actuarial molecular relapse rate at 24 months for the 2 groups is 91% and 33%, respectively ($P < 0.02$) (Fig 4). Ten patients, 9 with mixed T-cell chimerism, have relapsed (4 clinical, 6 cytogenetic) at a median of 12 months post-BMT.

**DISCUSSION**

This study has confirmed the high incidence (73% in this report) of mixed chimerism that has been seen after T-cell-
decreased incidence of residual disease in our study may be. If the same tolerance also applied to host myeloid cells, this would result in abrogation of any GVL effect resulting in an increased incidence of relapse. Because the removal of T-cells from the marrow graft is correlated with an increased incidence of relapse post-BMT, we decided to study T-cell chimerism post-BMT. Additional evidence that supports the role of the T cell in mediating GVL comes from studies using donor leukocyte infusions to induce remissions in patients with CML who relapse post-BMT.

The finding that mixed T-cell chimerism leads to an increased incidence of residual disease in our study may be related to immunologic tolerance. The presence of host T cells suggests that the donor T-cells may be tolerant to host T cells. If the same tolerance also applied to host myeloid cells including the residual leukemia cells, this would result in abrogation of any GVL effect resulting in an increased incidence of relapse.

It is well documented that GVHD is associated with a GVL effect and that patients who are full donor chimeras are more likely to develop GVHD. In our study, we have too few patients with GVHD to make any general comments on the link between chimerism, GVHD and GVL. Nevertheless, the data presented on unique patient no. (UPN) 1231 (Fig 3), a patient who was a complete donor chimera throughout her post-BMT course, and had a rising burden of residual disease while remaining on immunosuppressive drug therapy, which when discontinued, resulted in GVHD with a GVL effect and eradication of residual disease. It is possible that the cyclosporine and prednisone therapy led to a pharmacologically mediated immunologic tolerance that postponed an effective GVL response. If this hypothesis is valid, then it is possible that prolonged immunosuppressive therapy to prevent chronic GVHD after conventional BMT might be associated with a higher relapse rate in patients with CML.

The incidence of leukemia relapse after conventional BMT for CML is between 9% and 24%. Mixed chimerism has been reported in up to 44% of patients after conventional BMT when PCR-based methodologies are used. It is tempting to speculate that patients with CML who relapse after conventional BMT for CML belong to the group of patients who are mixed chimeric. There are currently no published data to address this issue.

There have been many studies on the prognostic significance of a positive PCR for BCR-ABL mRNA and subsequent relapse post-BMT. It has been reported that two sequential positive assays are predictive of relapse. The data that we present in this paper are consistent with that observation. It has been reported that PCR positivity within the first 6 to 9 months post-BMT may not be of prognostic significance because many of these patients eventually become PCR negative and have prolonged clinical remissions. These studies have largely involved patients who received conventional transplants. Our data suggest that a single PCR-positive result within the first 6 months of transplant after a T-cell-depleted BMT is predictive of subsequent cytogenetic or hematologic relapse. Although this data is contrary to what has been previously published after conventional BMT, it makes biologic sense and is compatible with the observation that T-cell depletion prevents an effective GVL response.

The finding that early BCR-ABL positivity after T-cell-depleted BMT has prognostic value could have direct therapeutic implications. One possible strategy might be to do T-cell-depleted BMT in patients with CML who were assessed to be at high risk of GVHD and transplant-related mortality. PCR for BCR-ABL mRNA could be used to determine if residual disease is present and the result used to initiate donor leukocyte infusion immunotherapy in patients with minimal residual disease. Early intervention might prevent the complication of pancytopenia seen with this treatment and might allow for substantially fewer donor leukocytes to be used, thereby potentially reducing the incidence of significant GVHD.

In summary, the use of molecular techniques to assess chimerism and residual disease allows us to have more insight into the processes that ultimately lead to cure after BMT for CML. The next step is to harness these techniques to develop new strategies for improving patient management.

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