Chronic Myelogenous Leukemia (CML) is a clonal proliferative disorder of hematopoietic stem cells characterized by the t(9;22) chromosomal translocation that forms the chimeric bcr-abl gene.1,2 Currently, high-dose myeloablative chemotherapy followed by allogeneic bone marrow transplantation (BMT) provides the most effective treatment for this disease with 40% to 60% of patients remaining disease-free more than 5 years post-BMT.3,5 Because CML cells are characterized by a unique tumor-specific gene, the sensitive polymerase chain reaction (PCR) can be used to detect residual bcr-abl mRNA expressing cells post-BMT.6-8 Several studies have shown that serial PCR analyses for bcr-abl-positive cells allow the identification of distinct patient groups with different clinical outcomes after allogeneic BMT for CML.3,11 In patients who remain persistently PCR positive post-BMT, it appears that neither the ablative regimen nor posttransplant factors such as graft-versus-host disease (GVHD) have effectively eliminated proliferating leukemic cells, and such individuals have a high risk of relapse. In contrast, patients who are persistently PCR-negative appear to have either eliminated or suppressed the malignant clone below the limit of detection by PCR and such individuals have an excellent prognosis. Interestingly, previous studies have also shown that some patients are intermittently PCR-positive and PCR-negative after BMT and these patients have an intermediate risk of relapse. Such individuals appear to harbor detectable residual bcr-abl-positive cells for extended periods of time reaching several years post-BMT, but many of these patients do not relapse and some eventually become completely PCR-negative.12-16 Why these patients do not relapse despite the apparent presence of leukemic cells post-BMT is an unsolved question.

Thus far, no studies have determined the lineage and proliferative potential of residual bcr-abl-positive cells in patients who have intermittently PCR-detectable cells after allogeneic BMT for CML. To better characterize residual bcr-abl-positive cells in such individuals, we examined individual colonies of myeloid progenitor cells obtained from five selected patients known to be intermittently PCR-positive or -negative at various times after allogeneic BMT. BM samples known to have PCR-detectable cells but without cytogenetic evidence of persistent or recurrent CML were cultured in vitro and individual myeloid progenitor cell colonies were analyzed for the presence of bcr-abl gene rearrangement by PCR.

MATERIALS AND METHODS

Patients and clinical samples. This study was performed in five patients with CML who underwent allogeneic BMT with CD6 T-
cell—depleted marrow at the Dana-Farber Cancer Institute (DFCI) in Boston, MA, between November 1987 and May 1992. Details of the BMT preparative regimen and method for T-cell depletion of donor marrow have been described previously. The BMT preparative regimen consisted of splenic radiation (750 cGy) in addition to cyclophosphamide (60 mg/kg x 2) and total body irradiation (Cy/TBI) (1.2 to 1.4 cGy). Marrow from HLA-identical sibling donors was treated in vitro with anti-T12 (CD6) monoclonal antibody (MoAb) and complement to selectively remove T cells, and no patient received prophylactic immune suppressive agents to prevent GVHD after BMT. All patients were treated according to clinical protocols approved by the Human Subjects Protection Committees at DFCI and informed consent was obtained from each individual. Heparinized peripheral blood (PB) and/or BM was obtained at different intervals post-BMT from these patients. The mononuclear cell fraction was isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque; Pharmacia, Piscataway, NJ) and cryopreserved in 10% dimethyl sulfoxide (DMSO).

**RNA extraction, reverse transcription, and double amplification PCR.** Total RNA was isolated from patient samples, normal individuals, and K562 cells using the guanidine thiocyanate method. Reverse transcription and double-amplification PCR was performed as previously described. Briefly 1 μg of total RNA was reverse transcribed to cDNA in a final volume of 20 μL, using 120 ng of Random Hexamer (Pharmacia), 500 umol/L of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI), 10 U of RNasin (Promega) and 100 U of MLV reverse transcriptase (Bethesda Research Laboratories [BRL], Gaithersburg, MD) in PCR-buffer (50 mmol/L Tris-CL, 2.25 mmol/L MgCl2, 0.01% gelatin, 4.5% NP 40, 4.5% Tween 20 [Sigma, St Louis, MO]). The reaction was performed at 42°C for 60 minutes and then at 95°C for 5 minutes. The first PCR amplification was performed in a volume of 50 μL using 10 μL of the reverse transcription mixture; 200 nmol of oligonucleotide; 200 umol of dATP, dGTP, dCTP, and dTTP; 1.25 U Taq Polymerase (Cetus, Emeryville, CA) in the same PCR buffer as used for reverse transcription. Reamplification of 1 μL of this amplified mixture was performed under the same conditions using oligonucleotide primers internal to the original primers. Ten-microliter aliquots of this final reaction were analyzed in a 2% agarose gel (Ultrapure agarose; BRL) containing 0.01% Ethidium bromide in Tris-borate electrophoresis buffer and visualized under UV light. Gels were then transferred to Nylon membranes (Zetaprobe blotting membranes; Biorad, Richmond, CA) and the presence of bcr-abl derived product was confirmed by hybridization with a specific 32P-labeled oligonucleotide. Two negative controls consisting of 1 μg of RNA, isolated from normal PB/MB cells and of sterile DEPC water (Diethylpyrocarbonate; Sigma Chemical, St Louis MO) including all reagents without RNA were performed in each experiment. To ensure that the desired sensitivity had been reached in each reaction, a positive control consisting of RNA from a 10−2 dilution of the cell line K562 in 1 μg RNA normal PB/MB cells was performed. Each positive sample was analyzed in at least two different aliquots and all PCR-negative samples were analyzed in at least four different aliquots. To ensure that RNA could be reverse transcribed and subsequently amplified, control amplification using abl sequence-specific primers was performed in all samples.

**Short-term methylcellulose assays.** Heparinized BM samples obtained from five patients and from five healthy individuals (negative controls) were cryopreserved in 10% DMSO after isolation of the mononuclear cell fraction by Ficoll-Hypaque density gradient centrifugation. Before culture, cells were thawed and washed three times in RPMI 1640 with 2.5% AB serum. A total of 5 x 105 cells were plated in 35-mm Petri dishes in 1 mL of culture mixture containing 0.9% methylcellulose (Dow A4 Premium; Dow, Midland MI), 30% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), 1% bovine serum albumin (BSA), 10−7 mol/L 2-mercaptoethanol, penicillin/streptomycin (Sigma), 2 U erythropoietin, 20 ng interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (all from Schering Corp, Kenilworth, NJ) and cultured at 37°C in a high-humidity 5% CO2 incubator. After 14 days, single erythroid burst-forming units (BFU-E), single granulocyte colony-forming units (CFU-G), and single macrophage colony forming units (CFU-M), each containing 50 to 100 cells, were removed using a disposable micropipette tip and directly added to guanidine isothiocyanate, to which 5 μg yeast RNA (Sigma) had been added as a carrier. RNA extraction, reverse transcription, and double-amplification PCR were then performed as described above.

**RESULTS**

**Patient clinical characteristics.** To identify and characterize the cells expressing chimeric bcr-abl mRNA identified by the PCR assay after allogeneic BMT, we examined BM samples from five selected patients. The clinical characteristics of these five patients are summarized in Table 1. All patients had received Cy/TBI followed by infusion of CD6 T-cell depleted BM from HLA-identical sibling donors. Four of the five patients had sex mismatched donors. Four of the five patients have remained in complete cytogenetic and clinical remission for prolonged periods (2 to 6 years) after BMT. One patient (no. 3) was found to have recurrent Ph+ recipient cells 3 years after BMT but has not yet developed any other evidence of relapsed CML. Only one of the patients (no. 5) developed chronic GVHD, which was not related to corticosteroid therapy. None of the other patients developed significant acute or chronic GVHD or received any immune suppressive therapy post-BMT. Four of the five patients (nos. 1 through 4) received low-dose recombinant IL-2 (rIL-2) for a 2 to 3 month period after engraftment with donor cells as previously described.

**Table 1. Clinical Summary of Patients Selected for Progenitor Cell PCR Analysis**

<table>
<thead>
<tr>
<th>Patient (UPN)</th>
<th>Age at BMT</th>
<th>Disease Status at BMT</th>
<th>Gender: Patient/Donor</th>
<th>Grade GVHD: Acute/Chronic</th>
<th>Cytogenetic Status</th>
<th>Clinical Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1599)</td>
<td>47</td>
<td>Stable</td>
<td>M/F</td>
<td>0/None</td>
<td>+24 mos normal male</td>
<td>+25 mos CCR</td>
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<tr>
<td>2 (1566)</td>
<td>41</td>
<td>Stable</td>
<td>F/M</td>
<td>0/None</td>
<td>+23 mos normal male</td>
<td>+28 mos CCR</td>
</tr>
<tr>
<td>3 (1471)</td>
<td>43</td>
<td>Stable</td>
<td>M/F</td>
<td>0/None</td>
<td>+35 mos mixed* chimera</td>
<td>+39 mos CCR</td>
</tr>
<tr>
<td>4 (1342)</td>
<td>30</td>
<td>Accelerated</td>
<td>M/F</td>
<td>0/None</td>
<td>+48 mos normal female</td>
<td>+53 mos CCR</td>
</tr>
<tr>
<td>5 (1162)</td>
<td>47</td>
<td>Stable</td>
<td>F/M</td>
<td>0/Limited</td>
<td>+56 mos normal male</td>
<td>+79 mos CCR</td>
</tr>
</tbody>
</table>

Abbreviation: CCR, complete clinical remission.

* Both Ph+ male cells and normal female metaphases detected.
PERSISTENCE OF bcr-abl+ PROGENITORS AFTER BMT

5
4
3
2
1

Fig 1. Results of serial PCR assays of blood and BM samples after allogeneic BMT. (●) PCR-positive result. (○) PCR-negative result.

PCR analysis of blood and marrow samples. Results of PCR analysis of mononuclear cells obtained from blood and/or marrow at various times after allogeneic BMT in all five patients are summarized in Fig 1. Each of these patients was found to be PCR-negative on at least two occasions after BMT. Each patient has also been PCR-positive on multiple occasions, but almost all samples in these individuals were PCR-positive only after the second amplification with nested primers. As shown in Fig 1, there was no consistent pattern of PCR reactivity in these individuals. For example, patient 5 remained consistently PCR-positive for almost 5 years but has subsequently been PCR-negative. In contrast, patient 4 was PCR-negative at 1 and 2 months post-BMT but has been consistently PCR-positive for over 4 years since that time.

PCR analysis of myeloid progenitor cell colonies. To determine both the lineage and proliferative capacity of PCR-positive cells detected after allogeneic BMT, single BFU-E, CFU-G, and CFU-M colonies were isolated from the BM of these patients and individual colonies were analyzed for the expression of hybrid bcr-abl mRNA. In each case, unfractonated BM mononuclear cells were known to be PCR-positive, but only after the second-step PCR amplification with nested primers. Results of PCR analysis of individual progenitor cell colonies in one patient (no. 4) are shown in Fig. 2. Thirty myeloid colonies were analyzed in a BM sample obtained 25 months post-BMT. PCR analysis showed that single erythroid, granulocytic, and macrophage colonies contained the bcr-abl gene rearrangement. Nevertheless, concurrent cytogenetic evaluation as well as subsequent examinations at 3 and 4 years post-BMT showed exclusively normal female donor metaphases. This patient remains in clinical hematologic remission 4 1/2 years after BMT and 2 1/2 years after PCR-positive myeloid progenitor cells were identified in his marrow.

Results of PCR analysis of myeloid progenitor cell colonies in all five patients are summarized in Table 2. In patient 1, PCR analysis of BM performed 5 months post-BMT showed the presence of bcr-abl transcript, but all 45 progeni-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample Date</th>
<th>BFU-E</th>
<th>CFU-G</th>
<th>CFU-M</th>
<th>PCR(^*) Colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+5 mos*</td>
<td>0/161</td>
<td>0/19</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>+5 mos</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
<td>3.3</td>
</tr>
<tr>
<td>3</td>
<td>+6 mos</td>
<td>0/10</td>
<td>1/10</td>
<td>1/10</td>
<td>6.6</td>
</tr>
<tr>
<td>4</td>
<td>+25 mos</td>
<td>1/10</td>
<td>1/10</td>
<td>1/10</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>+56 mos</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Months after BMT.

\(\dagger\) Values represent number PCR\(^*\) colonies/number colonies tested.

Fig 2. Example of an ethidium-bromide–stained agarose gel electrophoresis analysis of PCR products from single BFU-E, CFU-G, and CFU-M, respectively, from patient no. 4 including negative and positive controls. (a) bcr-abl–derived products; and (b) abl-derived products serving as controls indicating that RNA could be reverse transcribed and subsequently amplified in all samples. After double amplification, the bcr-abl PCR product consists of 290 bp in the presence of exon 3 (BFU-E, lane 4 and CFU-M, lane 8) or 217 bp in the absence of exon 3 (CFU-G, lane 6). The positive control consisted of single K562-derived colonies with 5 μg yeast RNA as a carrier, resulting in a 290-bp product. Negative controls included single BFU-E, CFU-G, and CFU-M from normal BM with 5 μg yeast RNA as a carrier and 5 μg yeast RNA only.
tor cell colonies derived from the same BM were PCR-negative. In patient 2, PCR analysis performed 5 months post-BMT detected bcr-abl-positive cells in both peripheral blood and BM. PCR analysis of 30 myeloid progenitor colonies grown from the same BM sample showed one bcr-abl mRNA-expressing macrophage colony. Cytogenetic analysis of BM cells obtained 1 1/2 and 2 1/2 years later (23 and 35 months post-BMT) continued to show only normal donor male metaphases without cytogenetic evidence of either recipient female or Ph+ cells. In patient 3, PCR analysis performed 6 months post-BMT was negative in peripheral blood, but positive in BM. PCR analysis of 30 myeloid colonies grown from the same BM sample showed one granulocytic and one macrophage colony that were PCR-positive. Subsequent cytogenetic evaluations of marrow obtained 11, 17, and 23 months post-BMT continued to show only normal donor female metaphases, but cytogenetic evaluation of marrow obtained 35 months after BMT again demonstrated Ph+ male cells as well as normal female metaphases. In patient 5, PCR analysis performed 56 months post-BMT was negative in peripheral blood, but positive in BM. In this patient, one bcr-abl-positive erythroid colony could be detected in 30 myeloid colonies that were analyzed. Cytogenetic analysis of the same marrow sample showed only normal male donor cells.

Overall, 7 of 135 progenitor cell colonies (5.2%) were found to be PCR-positive but expression of bcr-abl mRNA appeared to be equally distributed among committed erythroid, macrophage, and granulocyte progenitors. Despite the presence of bcr-abl-positive myeloid progenitor cells in four of the five patients, only one of these patients (no. 3) was subsequently found to have cytogenetic evidence of recurrent Ph+ cells. With additional follow-up of 20 to 33 months from the time of progenitor cell PCR analysis, none of these patients have yet developed clinical evidence of CML relapse.

**DISCUSSION**

In some patients with CML who have undergone allogeneic BMT, previous studies have shown that cells with bcr-abl gene rearrangement can intermittently be detected by the sensitive RT-PCR method many years after BMT without any sign of relapse. Because the bcr-abl oncogene is a unique and specific marker for CML and is presumably the causative abnormality in this leukemia, the clinical and biologic significance of this finding has not been adequately explained. It is conceivable that, because CML originates from a transformed pluripotent stem cell involving both the myeloid and lymphoid lineages, PCR positivity in such individuals might be caused by to the persistence of lymphocytes with the bcr-abl gene rearrangement that survived the BMT ablative regimen. These differentiated lymphoid cells would not be pluripotent and would therefore have less potential for inducing relapse. However, if residual PCR-detectable cells were found to be derived from the myeloid lineage and continued to have both the proliferative and differentiative capacities of CML cells, this would provide direct evidence that the PCR assay was, in fact, detecting leukemia cells fully capable of subsequently inducing relapse.

To directly examine the lineage and clonogenic potential of residual bcr-abl-positive cells, PCR was used to analyze individual myeloid progenitor colonies grown in a short-term methylcellulose assay for the expression of bcr-abl rearranged mRNA. These five patients were selected for further analysis because they remained in continuous clinical and cytogenetic remission for 2 to 6 years after allogeneic BMT for CML but had intermittently detectable bcr-abl-positive cells in their BM. The bcr-abl transcript was detected in myeloid progenitor cell colonies in four of the five patients. In one patient, no PCR-positive myeloid progenitors were detected even though analysis of BM, but not peripheral blood, showed the presence of bcr-abl rearranged cells. It cannot be excluded that bcr-abl rearranged cells consist solely of lymphocytes in this patient, but the fact that no bcr-abl rearranged cells could be detected in peripheral blood, where lymphocytes are more numerous than in BM, does not support this hypothesis. bcr-abl mRNA-expressing myeloid colonies may have been missed in this patient because of the relatively small number of progenitors analyzed or because minimal residual disease may be unevenly distributed in BM.

Overall, 5.2% of all progenitor cell colonies analyzed post-BMT were PCR-positive. Because unipotent progenitors from the erythroid, granulocytic, and macrophage lineages were found to have the bcr-abl gene rearrangement, the transformed cells must be at a relatively early stage of myeloid differentiation. The persistence of these cells in vivo for months to years after BMT coupled with the demonstrated ability of these cells to form colonies and differentiate in vitro clearly suggests that these cells are fully capable of self-renewal, proliferation, and myeloid differentiation. Nevertheless, our results also suggest that these cells are not able to proliferate and differentiate normally in vivo. Despite repeated examination of serial marrow samples, only one patient has had Ph+ cells detected by cytogenetic evaluation 2 1/2 years after PCR studies identified myeloid progenitor cells with the bcr-abl gene rearrangement. Although relatively few myeloid colonies were PCR-positive, an equivalent rate of differentiation of progenitors with the bcr-abl rearrangement in vitro should result in an equivalent fraction of mature myeloid cells with the same genetic abnormality. However, PCR assays were only positive after the second amplification step in all of these individuals and concurrent PCR analysis of blood samples were often negative. Because the first PCR amplification can usually detect 0.1% contamination with CML cells, PCR results in these five patients clearly indicated that far fewer than 5% of cells expressed bcr-abl mRNA. Moreover, 5% to 10% contamination by clonogenic cells with the bcr-abl gene rearrangement is also well within the limits of detection by conventional cytogenetic evaluation, but none of these patients were found to have Ph+ metaphases at the time of PCR analysis. Taken together, these results provide further evidence for the active inhibition of bcr-abl positive cells in vivo.

The application of BMT to the treatment of leukemia is
based in large part on the assumption that eradication of leukemia results primarily from the high-dose chemotherapy and radiation therapy administered pretransplant. However, substantial evidence now indicates that the transplantation of allogeneic BM also induces an antitumor effect, termed graft-versus-leukemia (GVL). The GVL appears to be an immunologic mechanism that is, in part, associated with both acute and chronic GVHD. Studies in experimental animal models and in clinical trials have shown that GVHD is mediated by mature T lymphocytes in the donor marrow inoculum. Experimental evidence suggests that donor T cells also mediate GVL and patients with CML who receive T-cell-depleted donor marrow have been found to have a higher risk of relapse after BMT. Nevertheless, other cells such as natural killer (NK) cells have also been implicated as possible mediators of GVL. Our demonstration that individuals who remain in remission with intermittently detectable bcr-abl rearranged cells harbor residual leukemic cells with clonogenic potential provides further indirect evidence for the presence of immunologic mechanisms capable of suppressing the proliferation of these cells for prolonged periods. Although the immunologic mechanism responsible for GVL in our patients has not yet been identified, only one of the five patients developed clinically apparent GVHD after transplant. Of interest, four of the five patients examined in this report also received prolonged low-dose IL-2 infusions post-BMT as part of another study to improve immune function and enhance GVL after T-cell–depleted allogeneic BMT. Previous experiments using a murine leukemia model have suggested that an operational cure of leukemia may be induced through amplification of natural host-defense mechanisms by IL-2 despite the continuous presence of clonogenic leukemia cells. Taken together, these findings support the hypothesis that GVL may also act independently from GVHD.

In summary, this report shows that long-term persistence of PCR-detectable minimal residual disease after allogeneic BM can be caused by the persistence of clonogenic myeloid precursors with the bcr-abl gene rearrangement that survived the BMT preparative regimen. Therefore, long-term remission and eventual cure may be achieved in some patients through continuous suppression of residual clonogenic leukemia cells. Identification of patients with such a GVL effect may enable us to identify the immunologic factors and cellular interactions that mediate GVL in vivo.

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Persistence of myeloid progenitor cells expressing BCR-ABL mRNA after allogeneic bone marrow transplantation for chronic myelogenous leukemia

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