Mixed hematopoietic chimerism (MC) is a common finding after allogeneic bone marrow transplantation (BMT), but the natural history of this phenomenon remains unclear. To understand the evolution and the implications of this finding, we performed a prospective analysis of the development of mixed chimerism in 43 patients with hematologic malignancies who received bone marrow (BM) from human leukocyte antigen (HLA)-identical sibling donors. T-cell depletion in vitro with anti-T12 (CD6) monoclonal antibody and rabbit complement was used as the only method of graft-versus-host disease (GVHD) prophylaxis. Overall, MC was identified in peripheral blood (PB) and BM in 22 of 43 (51%) patients evaluated. MC was found by restriction fragment length polymorphism (RFLP) analysis in 21 of 40 (53%) patients, by cytogenetic analysis in 6 of 29 (21%) patients, and by red blood cell phenotyping in 4 of 9 (44%) patients. RFLP studies were performed at 0.5, 1, 3, 6, 9, and 12 months post-BMT and then every 6 months, and showed a high probability of developing MC in the first 6 months after BMT followed by stabilization after 12 months. Cytogenetic analysis was less sensitive in detecting MC.

Although the transplant preparative regimens are thought to be ablative, there have been several previous reports demonstrating recovery of recipient hematopoiesis after BMT. These surviving recipient cells can either be normal hematopoietic host cells or tumor cells. When neoplastic cells are identified post-BMT, this often but not invariably results in clinical relapse. When these cells are normal recipient hematopoietic cells, they can either reject the donor bone marrow cells or can become tolerant to donor cells and contribute to the establishment of mixed hematopoietic chimerism. The phenomenon of graft rejection has been studied extensively both in experimental animal models and in clinical transplants, but the phenomenon of coexisting donor and recipient cells has only recently been recognized.

Several techniques have been used to evaluate mixed chimerism (MC) after allogeneic BMT. Cytogenetic analysis has been used frequently to differentiate between donor and recipient cells in patients with sex-mismatched donors; when donor and recipient are sex-matched, characteristic polymorphic regions or satellites can be used to distinguish the donor or recipient origin of the dividing cells. Donor and recipient red blood cells (RBCs) have been distinguished by analysis of surface antigens and enzymatic content. More recently, DNA analysis using Y-chromosome or probes for highly polymorphic regions on other chromosomes have been used to evaluate chimerism. These DNA restriction fragment length polymorphisms (RFLP) are presently used more often because they allow distinction and quantitation of donor recipient cells in almost all BMT patients and also because they can identify the origin of nondividing cells.

To clarify the clinical and immunologic implications of mixed chimerism, we have undertaken a prospective analysis of the development of mixed chimerism after allogeneic CD6-depleted BMT in 43 patients over a 5-year period. Overall, approximately 50% of these patients developed stable mixed chimerism after BMT. The probability of relapse, overall survival, and disease-free survival were identical for mixed chimeric patients with BM and CDH.
chimeras and patients who maintained complete donor hematopoiesis (CDH), but early reconstitution with T cells correlated with the subsequent maintenance of complete donor hematopoiesis.

**PATIENTS AND METHODS**

**Patients**

Between September 1983 and September 1988, 51 adult patients with various hematologic malignancies underwent allogeneic BMT using selective in vitro depletion of donor marrow with anti-T12 (CD6) monoclonal antibody (MoAb) and rabbit complement as the only method for prevention of graft-versus-host disease (GVHD). All patients and donors were matched at the human leukocyte antigen (HLA)-A, B, C, and D loci and were nonreactive in mixed lymphocyte culture. Clinical protocols were approved by the Institutional Review Boards of the Dana-Farber Cancer Institute and Brigham and Women's Hospital (Boston, MA) and BMT was performed at these two institutions with the informed consent of the patients. Overall, 43 of 51 patients could be evaluated prospectively for engraftment of donor cells and for return of normal recipient hematopoiesis after BMT. Mixed chimerism was evaluated in 14 patients by DNA analysis of restriction fragment length polymorphisms (RFLP) only, in 3 patients by cytogenetic analysis only, and in 26 patients by both methods. Eight patients were not evaluable for development of mixed chimerism for the following reasons: four patients died in the early posttransplant period before they could be evaluated for chimerism, three patients had either no pre-BMT sample or no adequate follow-up samples, and one patient manifested graft rejection within 3 weeks of BMT.

**Transplant Conditioning Regimen**

The conditioning regimen consisted of cyclophosphamide (60 mg/kg/d × 2) followed by fractionated total body irradiation (FTBI) delivered by a 4 mV linear accelerator (initially at 5 cGy/min and since 1985 at 10 cGy/min) for a total dose varying from 1,200 to 1,400 cGy in 6 to 7 fractions. Five patients received additional cytosine arabinoside (3 g/m2/12 h × 4) concurrently with cyclophosphamide, and one patient who had received prior mediastinal irradiation was treated with cyclophosphamide 60 mg/kg/d for 2 days and busulfan 4 mg/kg/d for 4 days. Immediately after BMT, BM mononuclear cells were isolated by Ficoll-Hypaque density sedimentation and treated with anti-T12 MoAb and rabbit complement as previously described.26

**Analysis of RFLP**

Before BMT, samples of peripheral blood or bone marrow from the patients and their donors were collected by preservative-free heparin and mononuclear cells isolated by Ficoll-Hypaque density sedimentation. In some instances, peripheral blood (PB) mononuclear cells obtained before BMT were cultured in vitro after stimulation with phytohemagglutinin (PHA) or allogeneic B-cell line (Laz 388). All cell samples were cryopreserved in 10% dimethyl sulfoxide (DMSO) using standard techniques and stored in the vapor phase of liquid nitrogen. After BMT, patient's BM and/or PB were collected at 0.5, 1, 3, 6, 9, and 12 months and then at 6-month intervals. Post-BMT samples were not stimulated or cultured in vitro before RFLP analysis.

Analysis of PB and BM samples to identify RFLP was performed by Collaborative Research, Lexington, MA, according to previously described techniques.26 High molecular weight (mol wt) DNA was isolated and digested with restriction endonucleases selected for their ability to allow recognition of highly polymorphic restriction fragments by defined probes. The digested DNA samples were size-fractionated by agarose gel electrophoresis and transferred to nylon membranes. Hybridization with selected 32P-labeled probes was followed by autoradiography. Post-BMT samples were analyzed simultaneously with mixtures of donor and recipient DNA to quantitate the relative amounts of donor and recipient DNA.

**Cytogenetic Analysis**

BM aspirates were performed 2 weeks after BMT and then every 6 months. Cytogenetic analysis was performed on BM cells after short-term culture (16 to 24 hours) without stimulation. In two instances only, patient's post-BMT PB lymphocytes were studied after stimulation with PHA for 72 hours. A mean of 29.2 (±8.0) metaphases were studied for each specimen using quinacrine banding. In 21 patients, sex chromosome determination allowed differentiation of donor and host cells. Autosomal heteromorphisms on chromosomes 1, 9, and 16 as well as D and G group chromosomes were used to differentiate between donor and recipient cells in six patients with sex-matched donors. Two patients with chronic myelogenous leukemia (CML), without any discriminating sex chromosomes or heteromorphism, had a (9;22) translocation that completely disappeared after BMT and reappeared 12 months later without evidence of clinical relapse. These two patients were considered cytogenetic relapses and mixed chimeras.

**RBC Typing**

RBC phenotype of recipient and donor PB was determined before BMT and differentiating markers of donor and recipient cells were identified in nine patients. RBC typing was evaluated every 3 to 6 months starting 4 months after transplant in patients who were not being transfused or were receiving blood transfusions negative for the markers. The percent donor and recipient RBC present was quantitated by differential agglutination using standard typing reagents (sensitivity to 5% recipient cells). Reverse grouping was used only when it indicated presence of donor or recipient isohemagglutinins, but the absence of ABO antibodies was not considered indicative of either mixed chimerism or complete donor hematopoiesis.

**Definition of Mixed Chimerism and Complete Donor Hematopoiesis**

Patients who demonstrated complete engraftment with donor cells and maintained 100% donor cells at all times until either last follow-up, relapse, or death were considered to have CDH. Patients who demonstrated presence of both recipient and donor cells in the BM or the PB by either RFLP, cytogenetics, or RBC typing at any time post-BMT were considered to have MC. All patients were evaluated clinically at least 2 months after their last sample, and no change in chimerism status was based on samples taken less than 4 weeks before the date of clinical relapse was considered for analysis. This was done to exclude cases of relapse with neoplastic recipient cells from the group of patients with mixed chimerism.

**Immunophenotypic Analysis**

Blood and BM samples were collected in preservative-free heparin and mononuclear cells were obtained after Ficoll-Hypaque density gradient sedimentation. Beginning 10 to 14 days post-BMT, blood was analyzed at weekly intervals in the first 4 to 6 weeks, then every 2 weeks for 1 month, and every month for up to 6 months. Cells were analyzed for reactivity with a panel of MoAbs: T1 (CD5), T3 (CD3), T4 (CD4), T8 (CD8), T11 (CD2), T12 (CD6), B1 (CD20), MY4 (CD14), and NKH1 (CD56) (Coulter Immunology, Hialeah, FL) by indirect immunofluorescence using standard techniques.27,28 Immunofluorescence reactivity was determined by automated flow cytometry.
Statistical Analysis

Differences in continuous variables after a normal distribution were analyzed with Student's t-test; the Mann-Whitney U-Test was used for variables not following a normal distribution. Fisher's exact test was used to test associations between categorical variables. Survival times, times to relapse, and times to chimerism were evaluated using Kaplan-Meier estimates and compared using the log-rank test.

RESULTS

Incidence of Mixed Chimerism After Allogeneic BMT

Evaluation of chimerism using RFLP analysis was obtained prospectively in 40 patients who underwent allogeneic BMT with T-12 depleted marrow from HLA-identical sibling donors. Patients were followed for up to 62 months after BMT, and within this group 21 patients (53%) were found to have mixed chimerism (Table 1). Two patients who were first found to have recurrent recipient cells less than 4 weeks before relapse were not considered to have mixed chimerism. Nineteen patients, who have been followed for a median period of 15.5 months, have maintained complete donor hematopoiesis.

In 29 patients, cytogenetic analysis could identify distinguishing markers between donor and recipient cells: six of these patients (21%) were found to have mixed chimerism. Thus, RFLP analysis detected mixed chimerism in a higher proportion of patients than cytogenetic studies \( P = .01 \).

Beginning 4 months after BMT, RBC typing was used to identify donor and recipient RBCs in nine patients: four patients were found to be mixed chimeras and five patients maintained complete donor hematopoiesis.

Probability of Mixed Chimerism

The probability of developing mixed chimerism at varying times post-BMT as determined by either RFLP or cytogenetic analysis is shown in Fig 1A. The probability of becoming a mixed chimera by RFLP analysis increases very rapidly in the first 6 months after BMT, reaching almost 50%. By 12 months post-BMT, the probability of being a mixed chimera plateaus at approximately 60% and remains stable for up to 4 years after BMT. In the 29 patients who were evaluable for chimerism by cytogenetic analysis, the probability of mixed chimerism against time is shifted to the right in comparison with the probability curve based on RFLP analysis, and the difference between the two curves is statistically significant \( P = .004 \). Instead of a rapid rise in the number of mixed chimeras during the first 6 months, cytogenetic analysis detected a gradual increase in the probability of mixed chimerism, which reached 33% at 14 months post-BMT and then stabilized at this level.

To further exclude the possibility that MC was identified because of sensitive detection of relapse, we also analyzed the probability of MC in the patients who have not relapsed post-BMT (Fig 1B). The probability of MC by RFLP again increases rapidly in the first 6 months after BMT and stabilizes at 0.48 after 9 months. In Fig 1B, the cytogenetics curve remains shifted to the right compared with the RFLP curve, but by 12 months after BMT the probability of detecting MC reaches 0.51, a probability level identical to that seen with RFLP.

Evolution of Mixed Chimerism

To evaluate the progression of mixed chimerism in our patients, we took advantage of the fact that RFLP analysis could not only determine the presence of recipient cells but could also allow estimation of the percentage of donor and recipient cells with a precision of \( \pm 5\% \) to 10% within PB and BM mononuclear cell populations.\(^{20-23}\) Mixed chimerism was detected as early in PB samples as in BM samples and differences between levels of MC were small. Since detection

<table>
<thead>
<tr>
<th>Table 1. Chimeric Status After Allogeneic BMT</th>
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<tr>
<td></td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>RFLP</td>
</tr>
<tr>
<td>Cytogenetics</td>
</tr>
<tr>
<td>RBC Typing</td>
</tr>
<tr>
<td>Total</td>
</tr>
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</table>

Abbreviations: MC, mixed chimerism; CDH, complete donor hematopoiesis; RFLP, restriction fragment length polymorphisms; RBC, red blood cell.

*RFLP versus cytogenetics: \( P = .01 \).
of MC and estimation of the percentage of recipient cells by RFLP analysis was as reliable if performed on PB samples as in BM samples, both types of results were compiled together. When both samples were evaluated simultaneously, the average of the percent recipient cells was used.

The percentages of recipient cells in mixed chimeras (Fig 2A) demonstrated a wide variation, but in this study there was no individual who reverted to complete donor hematopoiesis or progressed to 100% recipient cells at any time after the detection of mixed chimerism. The regression analysis shows a slow increase in the proportion of recipient cells with time after BMT. Only six patients became MC by cytogenetic analysis, and in these patients we also found a similar trend for an increase in the percentage of recipient cells with time (data not shown). To exclude the possibility that this slow increase in the percentage of recipient cells might be attributable to disease recurrence, we performed a similar analysis only with those patients who have remained in complete remission after BMT (Fig 2B). In these patients, we still find a slow and gradual increase from approximately 10% recipient cells 1 month posttransplant to approximately 50% recipient cells 40 months later.

During the course of this study, only three patients were found to have greater than 50% recipient cells at any time post-BMT. Interestingly, each of these increases was transient and all three patients are presently in clinical remission more than a year after having demonstrated more than 50% recipient cells. In one patient with acute myelogenous leukemia (AML), the percentage of recipient cells reached 75% at 3 months post-BMT. This sudden rise coincided with the development of a bacterial pneumonia and a significant decrease in the total white blood cell (WBC) count. Three months later, after treatment and resolution of this episode, the percent recipient cells was only 25%. The other two patients, one with acute lymphoblastic leukemia (ALL) and one with CML, have maintained 40% to 50% recipient cells and remain in clinical and cytogenetic remission.

Comparison of RFLP, Cytogenetics, and RBC Typing

To compare the ability of cytogenetics and RFLP to detect MC, we evaluated those patients who had both tests performed and excluded the three patients who could not be evaluated at the same time intervals. In this group of 23 patients, MC was detected by RFLP in 10 patients (43%); only five of these patients (22%) were detected by cytogenetic analysis and in no instance was MC detected by cytogenetics only. Thus, the sensitivity of cytogenetics compared with RFLP analysis is 50% but the specificity of cytogenetic analysis reaches 100%.

In the 23 patients who were evaluated with both methods, we also compared the percentage of recipient cells obtained by cytogenetics with the results obtained by RFLP analysis every time these tests were performed together in the same patients. In these instances, the mean percent recipient cells detected by cytogenetics (6.6%) was found to be significantly lower than by RFLP analysis (12.3%) \( (P < .05) \). Thus, RFLP studies detected mixed chimerism slightly more often and earlier, and found higher levels of recipient cells than cytogenetic analysis.

Among the 43 patients evaluated, the presence of donor and recipient RBC in PB could be evaluated in nine patients, and four were found to be mixed chimeras by RBC analysis. One patient had a Philadelphia chromosome detected 7 months after RBC chimerism was noted, and this cytogenetic relapse has since been followed for more than 18 months without evidence of clinical relapse. The other three patients (one patient with ALL and two patients with CML) remain in clinical and cytogenetic remission. All four of these patients who were classified as mixed chimeras by RBC typing had also been found to be mixed chimeras by RFLP. Of the five patients who had no recipient RBCs, one patient was identified as a mixed chimera by RFLP.

Influence of Patient Characteristics and Conditioning Regimen on the Subsequent Development of Mixed Chimerism

Patient and donor characteristics were evaluated to determine if they played a role in the subsequent development of mixed chimerism. As shown in Table 2, there were no differences in patient age, patient sex, donor sex, or donor-recipient sex match between the patients who became MC and the patients who maintained CDH. Patients with MC and CDH were divided equally among the different diagnostic categories and stages of disease except in the lymphomas, where none of the three patients (two patients with Hodgkin's
Table 2. Influence of Patient Characteristics on Subsequent Development of MC

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>MC</th>
<th>CDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
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<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Median age</td>
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<td>33</td>
<td>29</td>
</tr>
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<td>20-55</td>
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<td></td>
</tr>
<tr>
<td>F</td>
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<td>M</td>
<td>27</td>
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<tr>
<td>Donor sex</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>14</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>M</td>
<td>29</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>Donor/recipient sex match</td>
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<td></td>
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<tr>
<td>F/F</td>
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<td>1</td>
<td>2</td>
</tr>
<tr>
<td>M/M</td>
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<td>9</td>
<td>7</td>
</tr>
<tr>
<td>M/F</td>
<td>13</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>F/M</td>
<td>11</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AML</td>
<td>15</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>CML</td>
<td>16</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>ALL</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>MDS</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>First CR/stable phase</td>
<td>21</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Second or more CR-accel/blast</td>
<td>22</td>
<td>10</td>
<td>12</td>
</tr>
</tbody>
</table>

Abbreviations: AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CR, complete remission.

disease and one patient with non-Hodgkin's lymphoma) was found to be a mixed chimera.

The conditioning regimen consisted of FTBI and cyclophosphamide (CY) in 42 patients. Six patients received 1,200 cGy, and 5 of these patients received additional cytosine arabinoside, 10 received 1,300 cGy, and 26 had 1,400 cGy while CY dose remained stable at 120 mg/kg. The number of patients who were found to be mixed chimeras was the same in each treatment category including those that received higher doses of FTBI. Thus, in the range of doses given for the agents described above, there was no difference in development of mixed chimerism.

Influence of BM Graft Characteristics and Immunologic Reconstitution on Development of Mixed Chimerism

Characteristics of the donor graft were also studied to determine if they played a role in the development of mixed chimerism. The total number of BM cells, the number of cells/kg, the percentage and the total number of T cells and natural killer cells, namely of CD2 (T1), CD3 (T3), CD4 (T4), CD5 (T1), CD6 (T12), CD8 (T8), and NKH1 (CD56) infused were similar in patients with MC and CDH.

Immunologic reconstitution of T and NK cell subsets in patients with MC and CDH is shown in Fig 3. Significant phenotypic differences between patients with MC and CDH were only found in the first 2 weeks after BMT. At 2 weeks post-BMT, patients who later maintained CDH had 30.3% (±5.4% SEM) CD3+ cells, while patients who developed MC had an average of only 13.2% (±2.5% SEM) CD3+ cells (P = .008). The fraction of CD4+ cells was also significantly higher in the patients with CDH (24.1% ± 7.6%) than MC (8.7% ± 1.8%), but there was no difference in CD8+ cells. Similarly the CD2, CD5, and CD6+ cell populations were approximately 10% higher in the patients with CDH than in the patients with MC, but these differences did not reach statistical significance. By 4 to 6 weeks after BMT, the differences in percentage of all T-cell markers, including CD3 and CD4, disappeared between patients with CDH and MC. Within the first 8 weeks after BMT, NK cells were slightly lower in the patients with CDH than in the patients with MC, but the differences between these two groups was

![Fig 3. Immunologic reconstitution in the early post-BMT period. The number of peripheral blood CD3+, CD4+, CD6+ and NKH1+ cells after BMT was determined at various times by indirect immunofluorescence reactivity with specific MoAbs. Results in patients with MC (dashed line) are compared with results in patients with CDH (solid line).](image-url)
not statistically significant at any time. When we evaluated absolute numbers of T and NK cells after BMT, patients with CDH still maintained significantly higher numbers of CD3+ ($P = 0.03$) and CD4+ ($P = 0.04$) cells than patients with MC but differences for all other markers were not significant.

**Patient Outcome**

*Mixed chimerism and GVHD.* GVHD developed in only 5 of 43 patients studied. All five patients with acute GVHD were found to have CDH ($P < .02$) (Table 3). Two of these patients died 1.5 and 8.3 months after BMT, but the three other patients with GVHD could be followed for more than 2 years without any evidence of MC. One of these patients developed chronic GVHD.

*Mixed chimerism and relapse.* Within the 22 patients with MC, 11 have relapsed, whereas 6 of the 21 patients with CDH have relapsed; this difference is not statistically significant ($P = .22$). Kaplan-Meier estimates of relapse for patients with MC or CDH (Fig 4) showed that the majority of relapses occurred in the first year after BMT for both groups. The probability of relapse then stabilized at 0.60 for patients with MC (95% confidence interval [CI] ±0.25) and 0.42 for patients with CDH (95% confidence interval ±0.28). This difference is not statistically significant ($P = .29$). Neither the date of detection of MC, the proportion of recipient cells, or the duration of MC could help predict which patients were more likely to relapse.

*Mixed chimerism, survival, and disease-free survival.* Finally, 64% of the 22 patients with MC remain alive with a median follow-up of 11 months post-BMT. For the group of patients with CDH, the results are similar with 48% of the patients remaining alive. As shown in Fig 5A, the 4-year survival is estimated as 57.8% (CI: 34.3 to 81.2) for patients with MC and 50.8% (CI: 28.6 to 73.0) for patients with CDH, the median survival not being reached for either group of patients. Similarly, as shown in Fig 5B, the disease-free survival for patients with MC is estimated as 37.2% (CI: 13.1 to 61.3), which is almost identical ($P = .32$) to that of patients with CDH (39.7% with CI: 16.9 to 62.5).

**DISCUSSION**

In this study, 43 patients were prospectively evaluated for the development of mixed hematopoietic chimerism after allogeneic BMT. During a 5-year period of observation, mixed chimerism was identified in 22 patients, while 21 patients maintained complete donor hematopoesis. Since chimeric status was evaluated by both cytogenetic and RFLP analysis, we were able to compare the sensitivity of these two methods. We found that RFLP analysis detected mixed chimerism in 53% while cytogenetics detected mixed chimerism in only 21% of the patients evaluated by this method. There were no instances where mixed chimerism was detected by cytogenetics and not by RFLP. Thus RFLP was not only useful for detecting mixed chimerism in patients without sex-mismatched donors or cytogenetic polymorphisms, but this method also appeared to be more sensitive for detecting recurrence of recipient cells after BMT. Interestingly, as shown in Fig 1, the difference in the ability of RFLP and cytogenetics to detect mixed chimerism occurs primarily in the first year post-BMT.
There are several reasons that RFLP is a more sensitive method for detection of mixed chimerism in the early posttransplant period. First, each RFLP analysis includes DNA extracted from at least $10^6$ cells, while cytogenetics only evaluates 20 to 50 cells in each sample. DNA studies using RFLP can easily detect 5% to 10% chimerism, and under optimal conditions can detect 0.1 to 1% chimerism. On the other hand, the cytogeneticist is bound by the laws of probability and must examine 30 metaphases to exclude with 95% confidence that 1 cell in 10 is originating from the recipient, and 59 or more metaphases to exclude presence of 5% recipient cells. Second, RFLP analysis includes DNA from all nucleated cells, whether or not these cells are proliferating, while cytogenetic analysis evaluates only the fraction of cells that are spontaneously dividing. Nevertheless, in this study, the ability of chromosomal analysis to detect MC equaled RFLP in patients who did not relapse (Fig 1B). This finding may be attributable to the fact that these patients had a much longer follow-up than relapsed patients.

Despite the sensitivity of RFLP, it is nevertheless possible that more sensitive methods such as those based on detection of specific DNA sequences by polymerase chain reaction (PCR) might indicate that the true incidence of mixed chimerism is significantly above the 50% incidence established by RFLP. In our study, all cases of mixed chimerism were detected within the first 15 months post-BMT, and no patients who maintained complete donor hematopoiesis for 15 months have yet been found to have a return of normal recipient cells. This finding would suggest that mixed chimerism is an "all or none" phenomenon occurring in the first year after BMT. However, the demonstration of a relatively slow progression of mixed chimerism once it is identified also suggests that in those individuals with very few recipient cells (eg, <0.1%) it may take several years to achieve the 10- to 50-fold increase that would be required for detection by RFLP.

After allogeneic BMT, investigators have demonstrated that various hematopoietic lineages including marrow stromal cells, B lymphocytes, T lymphocytes, neutrophils, and erythrocytes can be of recipient origin. In our patients, chimerism was evaluated in mononuclear cells obtained from either PB or BM and purified populations were not studied. In almost all patients, RFLP analysis detected MC in PB, where the mononuclear cell fraction is predominantly composed of lymphocytes, as well as in the BM, where the majority of cells are myeloid. Cytogenetic analysis was performed almost exclusively in the BM and therefore detected MC primarily in dividing myeloid cells. MC was also detected in the RBCs of almost half the patients studied. Taken together, these results suggest that MC can be found as often in lymphoid as in myeloid or erythroid cells, and extends the findings in animal studies where MC was detected as often in PB as in BM. In future studies, it will be possible to analyze the origin of specific lymphoid subpopulations, including T-cell subsets, NK cells, and B cells.

In most previous studies, mixed chimerism has been evaluated over short periods of time and the natural history of mixed chimerism has not been established. To determine the kinetics of growth of both donor and recipient cells during this period of "cohabitation," we followed the percentage of recipient cells in all patients with mixed chimerism after BMT. We found that the extent of mixed chimerism varied widely but that there was a gradual increase in the number of recipient cells with time. Surprisingly, no patient with persistent recipient cells in the immediate post-BMT period was found to lose these cells afterwards. This finding shows that MC detected as soon as 2 weeks after BMT is significant when found in PB or BM, and does not result from persistence of dying cells. In patients with mixed chimerism, the percentage of host cells increased by approximately 20% over a 3-year period of observation. When we excluded those patients who relapsed to eliminate cases where tumor cells could have contaminated the BM and contributed to the detection of MC, we found the same upward trend in favor of recipient cells. It therefore seems that normal recipient cells have a proliferative advantage over donor cells, but this advantage is slight and both cell types are able to coexist and function in a relative state of equilibrium.

In previous studies, mixed chimerism was found to occur in 11% to 57% of patients who underwent allogeneic BMT without T-cell depletion, and in as many as 50% to 100% of patients who received BM grafts after in vitro T-cell depletion. This increased incidence of MC with T-cell depletion suggests that T cells may play a role in maintenance of CDH. In our study, immunologic reconstitution data showed that patients who maintained CDH had higher levels of T cells 2 weeks after BMT than patients who became MC. Among T cells, the percentage and absolute number of CD3+ and CD4+ cells demonstrated a significant difference between patients with CDH and MC. The higher number of CD4+ cells in patients with CDH suggests that recurrence of recipient cells is not only a function of the preparative regimen, but that helper T cells might play a role in preventing recipient cells from repopulating the patient's marrow and that presence of these cells is most important in the first weeks after BMT. Because all of the patients examined in this study engrafted with donor cells during this period, there is no evidence to suggest that the same cells that prevent the regrowth of recipient cells also promote donor engraftment.

In our study, all patients who developed GVHD also maintained CDH, suggesting that there might be a relationship between GVHD and the ability of the donor graft to suppress recipient hematopoiesis. Since donor T cells are responsible for GVHD, this finding is consistent with our observation that early T-cell engraftment is associated with maintenance of CDH. Nevertheless, only 12% of patients developed GVHD but 49% of patients maintained CDH. Animal studies have also provided evidence that a population of donor cells can help promote donor hematopoiesis without producing GVHD. Taken together, these results suggest that the T cells responsible for sustained CDH may be distinct from those T cells that cause GVHD.

Some studies indicate that mixed chimeras may be at higher risk of relapse, while other studies do not corroborate this finding. In this study, the probabilities of relapse as well as survival and disease-free survival post-BMT were similar in patients with MC and CDH.
These findings indicate that recurrence of normal recipient cells does not adversely affect the prognosis of BMT patients. It also suggests that the subset of cells responsible for the graft-versus-leukemia (GVL) effect is distinct from those cells that may be responsible for preventing recurrence of normal recipient hematopoiesis. In previous studies we found that donor NK cells are capable of cytolytic activity against residual leukemia cells; therefore we postulated that NK cells are more likely to mediate GVL than donor T cells. In contrast, our results suggest that NK cells are not involved in suppression of normal recipient hematopoietic cells.

One interesting implication of our study is that mixed hematopoietic chimerism appears to be an active phenomenon that results from the interactions of various subsets of donor and recipient cells. In future studies, it will be possible to use the sensitive detection and discriminating ability of RFLP to further characterize this phenomenon and define the immunologic mechanisms that either suppress or promote the reestablishment of recipient cells after allogeneic BMT. Moreover, when mixed chimerism develops it will be of interest to better define the ability of each population to become immunologically tolerant to the other and to determine if functional immunologic reconstitution is affected by this process.

ACKNOWLEDGMENT

We thank J. Fitzsimmons, K. Cochran, C. Ish, and J. Webb for technical assistance in the processing of patient specimens.

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