Analysis of the Origin of Marrow Cells in Bone Marrow Transplant Recipients Using a Y-Chromosome-Specific In Situ Hybridization Assay

By Diane M. Durnam, Kirk R. Anders, Lloyd Fisher, John O’Quigley, Eileen M. Bryant, and E. Donnall Thomas

A Y-chromosome–specific in situ hybridization assay was used to assess the frequency with which host bone marrow cells are retained after marrow grafting. The majority of patients (74%) showed the presence of both host and donor marrow cells when assayed 14 days after transplant. By 84 days posttransplant only 4% of the patients retained host marrow cells. Only 1 of 19 evaluable patients analyzed over 1 year posttransplant showed minimal retention of host cells. No statistical correlation was found between retention of host cells posttransplant and the development of relapse or acute or chronic graft-versus-host disease. Pretransplant conditioning regimen, HLA-matching, diagnosis, disease status at transplant, ABO-matching, and patient age also showed no correlation with the retention of host cells posttransplant.

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IN SITU HYBRIDIZATION POST-MARROW TRANSPLANT

Table 1. Clinical Characteristics

<table>
<thead>
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<th>Sex</th>
<th>Number</th>
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<tr>
<td>Female</td>
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<td>Male</td>
<td>39</td>
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<table>
<thead>
<tr>
<th>Patient Age (yr)</th>
<th>Median (range)</th>
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<tr>
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<td>33 (1-58)</td>
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<table>
<thead>
<tr>
<th>Diagnosis*</th>
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<tr>
<td>ALL</td>
<td>10</td>
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<td>Remission</td>
<td>7</td>
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<tr>
<td>Relapse</td>
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</tr>
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<td>ANL</td>
<td>9</td>
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<td>Remission</td>
<td>7</td>
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<tr>
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<td>CML</td>
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<td>Chronic phase</td>
<td>14</td>
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<td>Accelerated phase</td>
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<td>Blast crisis</td>
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<td>Juvenile</td>
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<tr>
<td>Relapse</td>
<td>7</td>
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<td>Multiple myeloma</td>
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<th>HLA matching</th>
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<td>Identical</td>
<td>50</td>
</tr>
<tr>
<td>Partially matched</td>
<td>17</td>
</tr>
</tbody>
</table>

| Patients receiving second transplants | 4 |

Abbreviations: ALL, acute lymphoblastic leukemia; ANL, acute non-lymphoblastic leukemia; CML, chronic myelogenous leukemia.

*Number of patients in relapse or remission at the time of transplant.

incubate on slides for 30 minutes at 37°C. Following rinsing in PBS, the slides were then incubated with a 1:200 dilution of biotinylated avidin (in albumin/PBS) at 37°C for 1 hour. Slides were rinsed in PBS, and incubated with a 1:1,500 dilution of FITC-labeled avidin in albumin/PBS at 37°C for 30 minutes. Following rinsing in PBS, cells were counterstained with Evans Blue and mounted in PBS. All avidin and biotin reagents were purchased from Vector Laboratories (Burlingame, CA). Data generated from samples yielding a minimum of 50 or more scorable cells are included in this report. A maximum of 739 BM-GRAN and 703 BM-MC were scored. For data analysis, Y chromosome positive cells were scored as male; Y chromosome negative cells were scored as female.

Cytogenetic analysis. Cytogenetic analysis of unstimulated bone marrow cells was conducted on approximately days 14, 21, 28, 56, and 84 posttransplant. Long-term follow-up patients were also examined cytogenetically. Chromosomes were prepared from direct and 24 to 48 hour cultures using standard techniques and examined after Q- or G-banding.22,23 Data generated from samples yielding five or more metaphases are included in this report. At early times posttransplant (before day 84) a mean of 17 to 20 (range 5 to 31) metaphases was examined. At long-term follow-up a mean of 18 metaphases was analyzed.

Statistical analysis. Bone marrow samples from normal males and females were analyzed using the in situ hybridization procedure in order to calculate a normal tolerance interval (ie, assay background). A tolerance interval for a probability distribution is an interval that contains with some level of certainty a fixed proportion of the probability distribution. For this study, tolerance intervals that contain 95% of the distribution from these normal individuals with 95% certainty were constructed. For males the data looked exponentially distributed and were modeled by first determining the upper bound of a one-sided 95% confidence interval for the exponential parameter and then determining the value that contained 95% of the probability for that distribution. The data for normal females showed a reasonably Gaussian distribution when the arcsine transformation was used for the percent of positive cells. The sample mean and variance were used together with tables for the construction of Gaussian tolerance intervals.

The predictive value of the assay for relapse, acute or chronic graft versus host disease (GVHD) and survival was evaluated using the proportional hazards regression model.24 A two sample Student’s t test was used to assess the relationship between pretransplant parameters (dichotomized) and the number of cells retained at the intervals examined posttransplant.

RESULTS

To test the sensitivity of the in situ assay and to determine the frequency of obtaining either “false negatives” (ie, male cells that fail to hybridize) or “false positives” (ie, female cells that show hybridization) we analyzed bone marrow samples from normal males or females (Fig 1). Samples from 11 males showed that, on average, 98.4% (range, 95.2% to 100%) of the cells score positively in this assay. Analysis of bone marrow from seven females showed only 0.85% (range, 0% to 1.3%) of the cells scoring as false positives. Statistical analysis of these data indicates that posttransplant samples must show less than 94.4% positive cells in male marrows or greater than 2.7% positive cells in female marrows to score outside of the background levels of this assay (95% confidence interval; see Materials and Methods).

Marrow cells from 67 patients were analyzed by both in situ hybridization and cytogenetic analysis during the first 84 days posttransplant. The in situ assay gave informative results for 90% of the patients examined on day 14. At later times (days 21, 28, 56, and 84) in situ hybridization gave results on 97% to 100% of the patients. Marrow hypocellularity and inadequate nuclear morphology were the primary causes of assay failure. Cytogenetic evaluation of the same patients was effective in 51% of the patients examined on day 14. It gave informative results in 85% to 92% of the cases examined at the later timepoints. Marrow hypocellularity and poor metaphase morphology prevented cytogenetic evaluation of the remaining cases. Comparison of transfusion schedules and assay timepoints showed that neither assay was affected by transfusions.

In situ hybridization data were collected on 58 patients who received untreated donor marrow and showed no evidence of relapse or engraftment complications during the first 84 days following transplant. Table 2 shows the average number of Y chromosome positive cells present after transplantation. The highest level of host cells was found on days 14 and 21. Thereafter, the number of host cells retained by most patients was close to or below background levels. The patient-to-patient variation observed at the earliest timepoints became minimal by day 28. In general, patients showed fewer host BM-GRAN than host BM-MC on days 14 and 21. This observation undoubtedly reflects the difference in lifespan of the two cell types. At later timepoints, the two cell fractions showed similar levels of host cells and the data were pooled.

Table 3 shows the fraction of patients who retained host cells at each timepoint. Males and females showed similar
levels of host cells at all assay points; however, due to differences in the background levels scored in males and females, more males than females scored positively for host cells during the early time intervals posttransplant (Fig 2). By day 28, the fractions of males and females showing host cells were identical. On day 56, only one patient (UPN 4099) had host cells. Subsequent examination on day 84 showed only donor cells in this patient. Likewise, only one of the 28 patients examined on day 84 was positive for host cells. This patient (UPN 4055) had shown only donor cells on days 21 and 56 and remains disease-free 11 months posttransplant. Both of these patients had barely detectable levels of host cells.

Table 3 shows a comparison of in situ hybridization data and data generated by cytogenetic examination. Only three patients showed cytogenetic evidence of host cells posttransplant. UPN 4203 showed 1 of 10 metaphases to be host on cytogenetic analyses on day 14. This patient showed high levels of host cells on that day by in situ analysis as well (68% BM-GRAN; 94% BM-MC). UPN 4273 showed cytogenetic evidence of host cells on days 14 and 56 posttransplant. The in situ hybridization level of host BM-GRAN was highest on day 56 (4%). However, both cell fractions at day 14 and day 56 BM-MC showed few (<2%) host cells by in situ hybridization. UPN 4103 showed 1 of 33 metaphases to be of host origin on day 56 posttransplant. No elevated levels of host

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**Fig 1.** Y chromosome specific in situ hybridization of male and female bone marrow cells. Male cells (A) and female cells (B) are shown. A fluorescent spot indicative of Y chromosome hybridization is apparent in male cells (arrow; A); female cells show no hybridization (B).

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**Table 2. Analysis of the Percentage of Y Chromosome Positive Cells Present Posttransplant**

<table>
<thead>
<tr>
<th>Interval Posttransplant (d)</th>
<th>Percentages of Y Chromosome Positive Cells (mean [range])</th>
<th>BM-MC</th>
<th>BM-GRAN</th>
<th>Total BM cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>77.8 (7.0-100)</td>
<td>83.4 (10.3-99.8)</td>
<td>79.5 (9.3-99.8)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>97.1 (85.0-100)</td>
<td>98.7 (95.0-100)</td>
<td>98.1 (93.2-99.7)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>99.4 (97.8-100)</td>
<td>99.2 (97.2-100)</td>
<td>99.2 (97.2-100)</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>99.2 (98.1-100)</td>
<td>98.8 (97.1-100)</td>
<td>99.0 (97.9-100)</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>99.3 (97.8-100)</td>
<td>98.6 (96.5-100)</td>
<td>98.7 (96.6-100)</td>
<td></td>
</tr>
<tr>
<td>Male patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>19.8 (0.6-94.3)</td>
<td>13.3 (0.4-68.4)</td>
<td>15.7 (0.5-87.3)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>4.9 (0-43.5)</td>
<td>2.1 (0-5.4)</td>
<td>4.3 (0-43.4)</td>
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</tr>
<tr>
<td>28</td>
<td>2.0 (0-8.7)</td>
<td>1.9 (0-8.1)</td>
<td>1.9 (0.2-8.5)</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>1.2 (0-4.6)</td>
<td>0.9 (0-3.2)</td>
<td>1.1 (0-3.9)</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>1.4 (0-4.6)</td>
<td>0.8 (0-2.4)</td>
<td>1.1 (0-3.5)</td>
<td></td>
</tr>
</tbody>
</table>

*Number of patients examined at each timepoint is shown in Table 3 and Fig 2. Data do not include four patients who relapsed <day 84, three patients who failed to engraft, or three patients who received T-cell-depleted marrows.

†Derived by pooling BM-MC and BM-GRAN data.
cells were detected by in situ hybridization. All three patients remain in clinical remission 203 to 308 days after transplantation.

In situ hybridization data on two patients (UPN 2616 and UPN 3433) who received T-cell depleted second marrow transplants was indistinguishable from that of patients who received nondepleted marrows. UPN 2616 had less than 3% host cells on days 14 and 21. No additional analyses were conducted. UPN 3433 showed 4.6% host cells on day 14 and 0.6% host cells on both days 21 and 84. A third patient (UPN 3953) who received T-cell depleted marrow for his first transplant was one of three patients who suffered graft failure (see below and Table 4).

Three patients failed to engraft and four patients relapsed during the 84-day study period. In the graft failures, elevated levels of host cells were detected by in situ hybridization at all timepoints analyzed (Table 4). Cytogenetic analysis also detected high levels of host metaphases in two of these patients. No metaphases were available for analysis on the third patient. Of the patients who relapsed during the study period, both in situ hybridization and cytogenetic analysis detected host cells at the onset of relapse in UPN 4157 (Table 4). Analysis of UPN 4045 at the time of relapse was hindered by the suboptimal quality of the bone marrow received for both types of analysis on day 56. However, both assays detected the return of host cells. Appropriate samples

were not available for analysis by either assay on the other two patients. Neither assay predicted relapse before clinical onset. To determine whether the level of host cells detected by in situ hybridization at early times posttransplant was predictive of relapse after day 84 posttransplant, we compared the average number of host cells retained by 11 patients who relapsed after day 84 with those who remained disease-free 6 to 13 months posttransplant. No statistically significant association between relapse and the retention of host cells at any of the assay timepoints was determined (all P values > .25).

The predictive value of the percent host cells on days 14, 21, 28, 56, and 84 for predicting subsequent occurrence of death, acute GVHD and chronic GVHD was also examined. Thirteen tests were run. The day 56 and 84 values were not used to predict subsequent acute GVHD because onset of acute GVHD occurred before these timepoints. All P values were > .15 except for the day 14 prediction of subsequent survival (P = .07). We also correlated the average number of host cells retained at each assay timepoint with pretransplant conditioning regimens, diagnosis, disease status at transplant, age, ABO-matching, and HLA-matching. No statistically significant correlations were detected (data not shown).

To assess the occurrence of host cells at long posttransplant intervals following marrow transplantation, we conducted in

<table>
<thead>
<tr>
<th>Interval Posttransplant (d)</th>
<th>Marrow Cellularity* (%)</th>
<th>In Situ Hybridization (No. Patients Showing Host Cells/Total No. Patients Examined)†</th>
<th>Cytogenetics (No. Patients Showing Host Metaphases/Total No. Patients Examined)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>24</td>
<td>24/37 (65%)</td>
<td>2/29 (7%)</td>
</tr>
<tr>
<td>21</td>
<td>42</td>
<td>7/38 (18%)</td>
<td>0/45 (0%)</td>
</tr>
<tr>
<td>28</td>
<td>66</td>
<td>4/34 (12%)</td>
<td>0/45 (0%)</td>
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<tr>
<td>56</td>
<td>77</td>
<td>1/21 (5%)</td>
<td>2/39 (5%)</td>
</tr>
<tr>
<td>84</td>
<td>79</td>
<td>1/28 (4%)</td>
<td>0/43 (0%)</td>
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</table>

*Average percent of normal cellularity.
†Data do not include four patients who relapsed < day 84, three patients who failed to engraft, or three patients who received T-cell depleted marrows.
‡Data from BM-MC and BM-GRAN fractions were pooled before analysis.

![Figure 2](image-url)

**Table 3. Number of Patients Showing Residual Host Cells Posttransplant**

![Graph A](image-url)

**Female Patients**

![Graph B](image-url)

**Male Patients**

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and Posttransplant Relapse

Marrow In Situ Hybridization Cytogenetics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Interval Posttransplant (d)</th>
<th>Marrow Cellularity* (%)</th>
<th>In Situ Hybridization (No. Y Chromosome Positive Cells/Total No. Cells Examined)</th>
<th>Cytogenetics (No. Host Metaphases/Total No. Metaphases Examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN 3953</td>
<td>M</td>
<td>14</td>
<td>ND</td>
<td>16/22 (73%)</td>
<td>12/20 (60%)</td>
</tr>
<tr>
<td>(graft failure)</td>
<td></td>
<td>21</td>
<td>10-20</td>
<td>623/631 (98%)</td>
<td>20/25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>0</td>
<td>505/517 (97%)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56</td>
<td>0</td>
<td>422/433 (97%)</td>
<td>IM</td>
</tr>
<tr>
<td>UPN 4289</td>
<td>M</td>
<td>14</td>
<td>5</td>
<td>509/513 (99%)</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>(graft failure)</td>
<td></td>
<td>21</td>
<td>&lt;5</td>
<td>308/313 (98%)</td>
<td>IM</td>
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<tr>
<td></td>
<td></td>
<td>28</td>
<td>0</td>
<td>111/125 (89%)</td>
<td>IM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>&lt;5</td>
<td>442/445 (99%)</td>
<td>IM</td>
</tr>
<tr>
<td>UPN 4157</td>
<td>M</td>
<td>14</td>
<td>NE</td>
<td>76/456 (17%)</td>
<td>0/20 (0%)</td>
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<tr>
<td>(relapsed day 84)</td>
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<td>21</td>
<td>ND</td>
<td>12/564 (2%)</td>
<td>0/20 (0%)</td>
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<tr>
<td></td>
<td></td>
<td>28</td>
<td>80</td>
<td>119/436 (27%)</td>
<td>0/16 (0%)</td>
</tr>
<tr>
<td></td>
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<td>56</td>
<td>100</td>
<td>275/340 (81%)</td>
<td>0/20 (0%)</td>
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<tr>
<td></td>
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<td>84</td>
<td>100</td>
<td>430/437 (88%)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td>UPN 4045†</td>
<td>F</td>
<td>14</td>
<td>40</td>
<td>361/401 (90%)</td>
<td>16/20 (80%)</td>
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<tr>
<td>(relapsed day 56)</td>
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<td>21</td>
<td>ND</td>
<td>266/301 (88%)</td>
<td>0/20 (0%)</td>
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<td>80</td>
<td>420/426 (99%)</td>
<td>0/20 (0%)</td>
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<tr>
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<td></td>
<td>56</td>
<td>ND</td>
<td>265/266 (100%)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td>UPN 4244</td>
<td>F</td>
<td>14</td>
<td>30</td>
<td>84/70 (100%)</td>
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<tr>
<td>(relapsed day 84)</td>
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<td>21</td>
<td>ND</td>
<td>9/1,112 (1%)</td>
<td>0/20 (0%)</td>
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<td>28</td>
<td>ND</td>
<td>0/20 (0%)</td>
<td></td>
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</tbody>
</table>

Abbreviations: ND, not done; NE, not estimated; IM, insufficient metaphases.

*Percent of normal cellularity.
†Patient had 70% blasts on day 56. Both the BM specimens analyzed by in situ hybridization and cytogenetics were of poor quality. Analysis of in situ hybridization results was inhibited by extraneous material on the slides. Poor metaphase morphology allowed only the determination that 16 of the metaphases were missing the Y chromosome. These cells were presumed to be of host origin.
‡Detected at autopsy.

Situ hybridization analysis on 20 patients returning for long-term follow-up examinations. Three patients showed weak or variable levels of hybridization in BM-MC and therefore only data from BM-GRAN is reported (Table 5). One female patient (UPN 3569) showed high levels of Y chromosome negative cells by in situ hybridization. Cytogenetic evaluation showed her cells to be of donor origin with a Y chromosome containing a major deletion. Of the remaining 19 patients, only one (UPN 2492) showed a level of host cells outside of the background levels. None of the marrows analyzed cytogenetically showed evidence of host cells (Table 5).

**DISCUSSION**

We have demonstrated the utility of a Y chromosome specific in situ hybridization assay for identification of donor or host cells following bone marrow transplantation. The in situ assay, although limited to recipients with donors of the opposite sex, offers several advantages over other routinely used techniques. The fact that over 200 cells are typically scored by the in situ assay makes it more sensitive than cytogenetic analysis in which approximately 20 cells are evaluated routinely. Unlike cytogenetic analysis, the in situ assay is not limited to analysis of dividing cells. The in situ assay frequently yields meaningful data when marrow hypopcellularity hinders cytogenetic analysis. Compared with RFLP analysis, the in situ assay requires smaller cell numbers, can be completed within 2 days rather than the minimum of 5 to 7 days required for Southern blotting and has a sensitivity greater than that routinely achieved by RFLP analysis. Finally, the in situ assay can assess the origin of all cells within a sample rather than distinct cell populations as are assayed with red blood cell antigens or white cell markers.

Loss of the Y chromosome in cells from older males or leukemic cells and constitutional variation in Y-heterochromatin represent potential limitations of the in situ technique. In the current study, one long-term patient showed faint, variable hybridization, which resulted from deletion of most of the heterochromatic region of the Y chromosome. It is essential, therefore, that specimens from male patients or donors and leukemic cells be tested either cytogenetically or by the in situ assay before transplant to ensure Y chromosome integrity.

Our in situ hybridization data show that by day 84 posttransplant the vast majority of patients have marrows entirely populated by donor cells. Patients evaluated more
patients who showed host cells. Approximately 40% of their cells continued to be present for more than 2 years in the marrow. The interval between transplant and the detection of host cells is important in determining the fraction of host/donor mixed chimerism following transplantation for aplastic anemia.5 Unlike the patients in the current report, the aplastic anemia patients had been prepared with CY and TBI. These reports indicate that the incidence of host cells after transplant in the first month posttransplant is not necessarily clinically detrimental.3,13,14 We found no statistical correlation between the number of host cells retained at any time posttransplant and the development of acute GVHD, chronic GVHD, or relapse. We also found no correlation between diagnosis, disease status, conditioning regimen, bloodgroup matching, patient age, or HLA-matching, and the retention of host marrow cells posttransplant. Previous studies have shown host/donor cell mixtures in patients receiving T-cell depleted transplants.26-32 Our data show that of the three patients who received T-cell depleted marrows, one showed elevated levels of host cells associated with graft failure. The other two patients were recipients of second transplants and showed host cell levels indistinguishable from patients who received untreated marrows.

In situ hybridization is useful in demonstrating graft failure because it documents the disappearance of donor cells and the increase of host cells. In subsequent studies, we have used the in situ assay to distinguish graft rejection from a poorly functioning graft in which the marrow cells are of donor origin. With regard to relapse, both cytogenetics and the in situ hybridization assays confirmed clinical relapse in two patients who relapsed within the first 84 days posttransplant. In this study neither assay predicted relapse at assay timepoints before clinical relapse. This could result from the infrequency with which samples were studied after day 28.

Our data are in agreement with reports from other centers, which indicate that the retention of host cells posttransplant is not necessarily clinically detrimental.3,13,14 We found no statistical correlation between the number of host cells retained at any time posttransplant and the development of acute GVHD, chronic GVHD, or relapse. We also found no correlation between diagnosis, disease status, conditioning regimen, bloodgroup matching, patient age, or HLA-matching, and the retention of host marrow cells posttransplant. Previous studies have shown host/donor cell mixtures in patients receiving T-cell depleted transplants.26-32 Our data show that of the three patients who received T-cell depleted marrows, one showed elevated levels of host cells associated with graft failure. The other two patients were recipients of second transplants and showed host cell levels indistinguishable from patients who received untreated marrows.

ACKNOWLEDGMENT

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