Effects of In Vitro Depletion of T Cells in HLA-Identical Allogeneic Marrow Grafts

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We report results of a pilot study designed to evaluate the effects of in vitro depletion of T lymphocytes from donor marrow in patients receiving HLA-identical marrow grafts for treatment of hematologic malignancies. Twenty patients aged 31 to 50 years were prepared for transplantation with cyclophosphamide (120 mg/kg) and fractionated total body irradiation (12.0 or 15.75 Gy). All received cyclosporine after grafting. The donor marrows were treated with a mixture of eight murine monoclonal antibodies and rabbit serum complement in a manner that achieved a 2- to 3-log depletion of T cells in most patients. Initial engraftment occurred promptly in 19 of the patients, and only three had clinically significant acute graft-versus-host disease. Depletion of donor T cells, however, was associated with an increased incidence of graft failure, which occurred as late as 244 days after transplantation. Graft failure was transient in one patient but apparently was irreversible in seven others. Three of the seven patients had cytogenetic but not morphological evidence of leukemic relapse at the time of graft failure. All seven patients with irreversible graft failure have died, six after receiving second bone marrow transplants. Seven of the eight cases of graft failure occurred among the 11 patients prepared for transplantation with 12.0 Gy of total-body irradiation, and only one occurred among the nine patients with advanced malignancies who received 15.75 Gy of total-body irradiation. This association with irradiation dose suggests that host factors were partly responsible for the graft failures. Because graft failure seldom occurs in irradiated recipients of unmodified HLA-identical allogeneic marrow transplants, it appears that T cells in the donor marrow may serve a beneficial function in helping to maintain sustained engraftment possibly by eliminating host cells that can cause graft failure. Optimal application of in vitro manipulation of donor marrow as a method for preventing graft-versus-host disease will require more effective immunosuppression of the recipient in order to assure sustained engraftment and function of donor stem cells.

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Allogeneic bone marrow transplantation can provide curative therapy for patients with acute leukemia or aplastic anemia. However, clinically significant acute graft-versus-host disease (GVHD) occurs in 35% to 50% of patients who receive an allogeneic marrow graft from a genotypically HLA-identical sibling even though these patients are treated after transplantation with immunosuppressive agents such as methotrexate, cyclosporine, corticosteroids or antithymocyte globulin. The development of moderate (grade II) or severe (grade III or IV) acute GVHD after marrow transplantation is associated with decreased survival. At least 15% of marrow transplant patients die of complications related to acute or chronic GVHD, indicating a clear need for more effective methods for preventing or treating this complication.

In numerous rodent models it has been demonstrated that mature T lymphocytes are responsible for causing GVHD after allogeneic marrow or spleen cell grafting. GVHD does not occur in irradiated animals when the graft is devoid of mature T cells. Transplanted fetal liver cells or spleen cells from neonatally thymectomized mice can reconstitute normal hematopoiesis without causing acute GVHD. Furthermore, removal of mature T cells from allogeneic donor spleen cells with the use of anti-Thy-1 heteroantisera or monoclonal antibodies can prevent GVHD. As few as $3 \times 10^8$ total T cells can cause GVHD in recipient mice with disparity only in non-H-2 minor histocompatibility antigens. Prevention of GVHD by removal of mature T cells has also been demonstrated in other species.

Removal of T cells from human donor marrow was first attempted with the use of a carefully titrated heterologous rabbit anti-T cell antiserum that was extensively absorbed in order to remove antibodies that were cross-reactive with hematopoietic precursors. Physical separation of T cells from the use of lectin agglutination and E rosetting has also been investigated. More recent attempts to remove T cells from human donor marrow have used murine monoclonal antibodies specific for surface molecules carried by human T lymphocytes. Previous studies have demonstrated that treatment of marrow with unmodified murine monoclonal antibodies in the absence of exogenous complement does not prevent GVHD.

We report here results of a pilot study designed to evaluate the effects of in vitro depletion of T lymphocytes from donor marrow with the use of anti-T cell monoclonal antibodies and complement. The objectives of this study were to determine (a) the toxicity of marrow treatment, in terms of delay or failure of engraftment; and (b) the efficacy of T cell depletion as a method of preventing acute GVHD. Patients in this study received genotypically HLA-identical marrow grafts for treatment of hematologic malignancies, and results for engraftment and acute GVHD were compared with those for similar patients who received unmodified donor marrow.

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Graft recipients more than 30 years of age were chosen because of their greater incidence of GVHD and, because the mortality of the disease is highest in these patients. The 2- to 3-log T cell depletion achieved with a mixture of monoclonal antibodies and complement was sufficient to decrease the incidence and severity of GVHD but was associated with an increased incidence of graft failure and poor survival.

MATERIALS AND METHODS

Bone marrow transplantation protocols. Patients in this study were age 30 or older, had hematologic malignancies, and received marrow from HLA genotypically identical sibling donors. Patients were prepared for transplantation with cyclophosphamide, 60 mg/kg/d on each of two consecutive days followed by total-body irradiation delivered from dual opposing 60Co sources at a dose rate of 8.0 R/min (measured at the midplane in air). Patients with chronic myelogenous leukemia in chronic phase or acute nonlymphocytic leukemia or lymphoma in remission received a total of 12.0 Gy delivered in fractionated doses of 2.0 Gy on each of six consecutive days. Patients with more advanced disease received a total of 15.75 Gy delivered in fractionated doses of 2.25 Gy on each of seven consecutive days. Intrathecal methotrexate (12 mg) was administered twice during the week before marrow infusion.

All patients received cyclosporine, 3.0 mg/kg/d intravenously (IV) in two divided doses, for prophylaxis of GVHD beginning on the day before marrow infusion. When oral medication could be tolerated, cyclosporine, 12.5 mg/kg/d PO, was administered in two divided doses. The dose of cyclosporine was reduced in patients with impaired renal function. Beginning on day 50 after transplantation, the dose was gradually decreased by 5% per week, and treatment was discontinued six months after transplantation. As prophylaxis for leukemic relapse in the central nervous system, intrathecal methotrexate (12 mg) was administered every other week, beginning on day 32, for a total of six doses. Six hours after each dose of intrathecal methotrexate, patients received leukovorin, 12 mg IV or PO every six hours for a total of eight doses. All patients were entered on one or more protocols for evaluation of infection prevention, including placement in a protective environment, prophylactic granulocyte transfusions, or prophylactic systemic antibiotics.

Engraftment was demonstrated by marrow examination and by rising peripheral white cell counts. Where possible, the presence of donor cells was demonstrated by cytogenetic studies or by blood genetic markers. The criteria for diagnosis and staging of acute GVHD have been described. Final assessment of GVHD was based on review of flow sheets, biopsy reports, and discharge summaries. The procedures, along with the potential risks and benefits, were explained in detail to patients and family members. All protocols were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Antibodies. A mixture of eight murine anti-T cell monoclonal antibodies was used for treatment of donor marrow (Table 1). Antibody 35.1 reacts with the E rosette receptor of human T lymphocytes, and antibodies 10.2, 38.1, 64.1, and 12.1 react with other well-defined "pan-T" antigens. Antibodies 9.3, 66.1, and 51.1 react with subsets of human T cells (reviewed27). With one exception, these antibodies bind noncompetitively (antibody 64.1 blocks the binding of antibody 38.1 but not vice versa). Thus the mixture was selected in order to provide optimal antibody binding to all T lymphocytes. Antibodies of the IgG class were purified from ascites fluid by solid phase adsorption on staphylococcal protein A columns. Antibodies of the IgM class were purified from ascites fluid by euglobulin fractionation. Each antibody was tested for purity (by microzonial electrophoresis), immunoglobulin concentration (by radioimmunoassay), and binding activity. Antibodies diluted in normal saline containing 10% human serum albumin were mixed, filter sterilized, dispensed in sterile vials, and frozen at −80 °C. Aliquots of the antibody mixture were tested for sterility and endotoxin (by limulus assay).

Treatment of donor marrow. Marrow was aspirated from the iliac crests of the donor, screened to remove fat and particulate matter, and placed in a sterile transfusion transfer bag. The marrow was dispensed in 50-mL centrifuge tubes and diluted with an equal volume of saline containing 0.1% human serum albumin, penicillin, 100 U/mL (omitted if the recipient was known to be penicillin allergic), and streptomycin, 1 μg/mL (saline-HSA). Gravity-fed 15-mL underlayers of Ficoll-Hypaque (S.G. 1.077) were formed, and cells were centrifuged at 900 g for 20 minutes at 22 °C. Interphase mononuclear cells were collected and washed three times in saline-HSA. Cells trapped in the fat were recovered by repeated incubations at 37 °C. Washed pooled cells were incubated in a 50-mL tube with antibodies, each at saturating concentration, for 30 minutes at 4 °C. Cells (3.3 × 106) were washed twice in cold saline-HSA, resuspended in a volume of 10 mL, and incubated for 60 minutes at 37 °C with 45 mL undiluted rabbit serum (as a source of complement) containing DNase 1, 40 U/mL (Worthington, Freehold, NJ). The incubation with rabbit serum complement was repeated with a second 45-mL vol of rabbit serum, and cells were then washed three times in saline-HSA, resuspended to a vol of 50 mL, and infused into the patient. A single lot of prescreened adult rabbit serum (Pel-Freez, Rogers, Ark) was used for all marrow treatments. Immediately before use the rabbit serum was centrifuged at 27,000 g for 30 minutes at 4 °C and sterilized by passage through a 0.45-μm membrane filter (Nalgene, Rochester, NY).

Hematopoietic precursors were cultured from aliquots of unmodified bone marrow cells and Ficoll-Hypaque-purified bone marrow mononuclear cells before and after treatment with antibodies and complement. These assays were carried out in the laboratory of Dr Beverly Torok-Storb. Procedures for enumeration of committed immature erythroid precursors (burst-forming units [BFU-E]) and committed myeloid colony-forming units (CFU-GM) have been described in detail elsewhere. Briefly, BFU-E were grown in 0.15-ML plasma clots containing purified human urinary erythropoietin, 2.0 U/mL (specific activity > 100 U/mg protein, Terry Fox Laboratory, Vancouver, BC), and M0-conditioned medium, 40 μL/mL (generously provided by Dr David Golde, UCLA), as a source of burst-promoting activity. CFU-GM were grown in 0.3% agar containing 20% fetal bovine serum and placent-conditioned medium, 50 μL/mL, as a source of colony-stimulating activity.

The efficiency of T cell depletion was assayed by culturing aliquots of treated and untreated marrow mononuclear cells (105) at

| Table 1. Antibodies Used for Treatment of Donor Marrow |
|-----------------|-----------------|-----------------|
| Antibody* | Immunoglobulin | T Cell Molecule Recognized† | Equivalent Antibodies |
| 35.1 | IgG2 | CD2 | Leu5, OKT11, 9.6 |
| 10.2 | IgG2 | CD5 | Leu1, OKT1, T101 |
| 38.1 | IgM | CD3 | Leu4, OKT3 |
| 64.1 | IgG2 | CD3 | Leu4, OKT3 |
| 12.1 | IgG2 | CD6 | T12 |
| 9.3 | IgG2 | Tp44 | None known |
| 66.1 | IgM | CD4 | Leu3, OKT4 |
| 51.1 | IgG2 | CD8 | Leu2, OKT8 |

*See reference 29 for review.†See reference 30 for review.
of these control patients were identical to those used for the patients receiving cyclosporine administration, all treatment protocols used for the care of these patients also received a loading dose of 1.25 mg/kg intramuscularly on day 2. All patients over age 30 referred to this center who received HLA genotypically identical allogeneic bone marrow for treatment of hematologic malignancies and cyclosporine for prevention of GVHD were evaluated as external controls. Except for the differences in routes of administration, this resulted in comparable mean trough serum levels of cyclosporine (data not shown). Except for the differences in routes of administration, this resulted in comparable mean trough serum levels of cyclosporine (data not shown).

T cell blasts were enumerated by flow micromfluorometry (FACS IV, Becton Dickinson, Mountain View, Calif). This assay can reliably detect as few as 0.03% viable T cells present in bone marrow.

**External control patients.** All patients over age 30 referred to this center who received HLA genotypically identical allogeneic bone marrow for treatment of hematologic malignancies and cyclosporine for prevention of GVHD were evaluated as external controls for the comparison of engraftment and acute GVHD. The donor marrow transplants for the study patients contained a mean of 3.5 (range, 2.0 to 5.6) $\times 10^8$ nucleated cells per kilogram recipient weight. Density gradient separation of the marrow resulted in a substantial loss of nucleated cells accompanied by enrichment of committed erythroid and myeloid precursors assayed as BFU-E and CFU-GM (Table 3). Treatment of the marrow mononuclear cells with antibodies and complement resulted in a further loss of nucleated cells accompanied by a minor loss of BFU-E but no apparent effect on CFU-GM. There was wide variation in the percentage of recovery of BFU-E and CFU-GM, possibly reflecting dilution errors caused by cell clumping. Patients received less than 20% of the original number of nucleated cells, but these were more than fourfold enriched for BFU-E and more than threefold enriched for CFU-GM.

37°C for seven days in RPMI 1640 medium containing 15% pooled human serum and phytohemagglutinin (PHA), 1 μg/mL. Viabe cells were stained by indirect immunofluorescence using the anti-T cell antibody mixture at saturating concentrations. Brightly stained T cell blasts were enumerated by flow micromfluorometry (FACS IV, Becton Dickinson, Mountain View, Calif). This assay can reliably detect as few as 0.03% viable T cells present in bone marrow.

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**RESULTS**

**Patient characteristics.** The ages and diagnoses of the 20 patients in the study group and the 65 external control patients who received unmodified donor marrow were similar (Table 2). Approximately one third of the patients in both groups were placed in sterile laminar airflow isolation and received sterilized food and nonabsorbable oral antibiotics. A greater proportion of patients in the study group had sex-matched donors. The marrow transplants for the study patients were performed between June 24, 1983, and March 16, 1984. The marrow transplants for the control patients were performed between Oct 21, 1980, and Feb 10, 1984, with ten of the 65 performed after June 24, 1983. Results in both groups were evaluated as of Nov 1, 1984, with a minimum follow-up of 225 days.

**Marrow treatment.** Before treatment the harvested donor marrow for the 20 study patients contained a mean of 3.5 (range, 2.0 to 5.6) $\times 10^8$ nucleated cells per kilogram recipient weight. Density gradient separation of the marrow resulted in a substantial loss of nucleated cells accompanied by enrichment of committed erythroid and myeloid precursors assayed as BFU-E and CFU-GM (Table 3). Treatment of the marrow mononuclear cells with antibodies and complement resulted in a further loss of nucleated cells accompanied by a minor loss of BFU-E but no apparent effect on CFU-GM. There was wide variation in the percentage of recovery of BFU-E and CFU-GM, possibly reflecting dilution errors caused by cell clumping. Patients received less than 20% of the original number of nucleated cells, but these were more than fourfold enriched for BFU-E and more than threefold enriched for CFU-GM.

Before treatment with antibodies and complement, the marrow mononuclear cells for the 20 patients contained a mean of 1.10 (range, 0.49 to 2.44) $\times 10^9$ T lymphocytes identified by indirect immunofluorescence and flow microfluorimetry. After treatment, T cell blasts could not be detected in 15 of the 20 treated marrow samples cultured for seven days in medium containing PHA (Fig 1, Tables 4 and 5). Given the total number of viable nucleated donor cells infused and the threshold sensitivity of this assay (0.03%), these patients must have received fewer than 2.3 $\times 10^7$ T cells. Thus treatment with antibodies and complement resulted in at least 2.2 to 3.2 logs of T cell depletion for the 15 marrows in which PHA-driven T cell blasts could not be detected. Because the PHA-driven T cell assay is not quantitative once a threshold is exceeded, the extent of T cell depletion could not be reliably assessed in the five marrows in which T cell blasts were detected. Infusion of antibody and complement-treated marrow was well tolerated and without significant toxicity in any of the 20 patients.

**Initial engraftment.** Prompt engraftment was achieved in 19 of the 20 patients who received T cell-depleted marrow.

### Table 2. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Study Patients (n = 20)</th>
<th>External Controls (n = 65)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range), yr</td>
<td>35 (31–50)</td>
<td>36 (30–49)</td>
</tr>
<tr>
<td>Mean age, yr</td>
<td>37.5</td>
<td>36.5</td>
</tr>
<tr>
<td>Diagnoses, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute nonlymphocytic leukemia, remission</td>
<td>3 (15)</td>
<td>17 (26)</td>
</tr>
<tr>
<td>Acute leukemia, relapse</td>
<td>3 (15)</td>
<td>20 (31)</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia, chronic phase</td>
<td>7 (35)</td>
<td>19 (29)</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia, accelerated phase or blast crisis</td>
<td>6 (30)</td>
<td>9 (14)</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma, remission</td>
<td>1 (5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Laminar airflow isolation, n (%)</td>
<td>7 (35)</td>
<td>23 (35)</td>
</tr>
<tr>
<td>12.0 Gy total-body irradiation, n (%)</td>
<td>11 (55)</td>
<td>36 (55)</td>
</tr>
<tr>
<td>15.75 Gy total-body irradiation, n (%)</td>
<td>9 (45)</td>
<td>29 (45)</td>
</tr>
<tr>
<td>Sex-matched donor, n (%)</td>
<td>14 (70)</td>
<td>23 (35)</td>
</tr>
</tbody>
</table>

### Table 3. Cell Recoveries and Numbers Infused

<table>
<thead>
<tr>
<th></th>
<th>Mean Percentage of Recovery (Range)</th>
<th>Number of Cells Infused per kg (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1: Density Gradient Separation</td>
<td>Step 2: Antibody and Complement Treatment</td>
<td>Net Recovery</td>
</tr>
<tr>
<td>Nucleated cells</td>
<td>28 (14–46)</td>
<td>75 (56–98)</td>
</tr>
<tr>
<td>BFU-E</td>
<td>105 (27–246)</td>
<td>33 (46–142)</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>66 (17–137)</td>
<td>97 (63–127)</td>
</tr>
</tbody>
</table>

The mean percentage of cell recoveries after density gradient separation (step 1) and after antibody-complement treatment (step 2) are indicated separately. The final mean percentage of cell recoveries after separation, treatment, and washing is indicated as Net Recovery.
T CELL-DEPLETED MARROW GRAFTS

![Fluorescence histograms of untreated and treated donor marrow cells after culture for one week in PHA and staining with control antibody or anti-T cell antibodies. The equivalent fluorescence of treated cells stained with anti-T cell antibodies and with control antibody indicated that T cell blasts were not present.](image)

Fig 1. Fluorescence histograms of untreated and treated donor marrow cells after culture for one week in PHA and staining with control antibody or anti-T cell antibodies. The equivalent fluorescence of treated cells stained with anti-T cell antibodies and with control antibody indicated that T cell blasts were not present.

One patient had evidence of engraftment on day 14 but developed graft failure by day 21 and thus did not engraft fully (see later). Among the other 19 patients, granulocytes reached levels ≥1,000/mm³ at a median of 21 days, identical to granulocyte engraftment in the controls who received unmodified marrow (Fig 2). Engraftment of megakaryocytes occurred promptly in the study patients, and platelet transfusion support was terminated at a median of 19 days (range, 12 to 24 days). Engraftment of lymphocytes and leukocytes, however, was somewhat delayed compared with controls.

Graft failure before day 100. Three of the study patients (UPN 2248, UPN 2157, and UPN 2343) had graft failure on days 21, 55, and 69, respectively, as evidenced by the development of pancytopenia and marrow aplasia (Table 4). All three had been prepared for transplantation with 12.0 Gy of fractionated total-body irradiation, and two of the three (UPN 2343 and UPN 2157) had received transfusions before transplantation. Reappearance of host cells in the blood and marrow at the time of graft failure was demonstrated in UPN 2248 and UPN 2157. After reconditioning with cyclophosphamide and antithymocyte globulin, UPN 2248 received unmodified marrow from the original donor but died of systemic candidiasis seven days later, before engraftment could occur. Successful initial engraftment occurred in UPN 2157 after receiving a similar reconditioning regimen and unmodified marrow from the original donor, but the graft again failed and the patient died of hemorrhage. A second transplant was attempted without reconditioning in UPN 2343 using unmodified marrow from the original donor, but there was no evidence of engraftment 18 days later. Subsequently, the patient engrafted after reconditioning with cyclophosphamide, one dose of antithymocyte globulin, and infusion of unmodified marrow from a second HLA-identical sibling. This patient later developed three-system GVHD and died of CMV interstitial pneumonia on day 80 after the third marrow transplant. One additional patient (UPN 2299) had evidence of mixed chimerism on day 52 after transplantation (Table 1), but there was no evidence of graft failure.

Graft failure after day 100. On long-term follow-up, two additional study patients had unexplained difficulties with hematopoietic function (Table 4). Both had been prepared for transplantation with 12.0 Gy of fractionated total-body irradiation, and one had received transfusions before transplantation. UPN 2123 had a myeloid maturation arrest diagnosed on day 148, and on day 153 his granulocyte count reached a nadir of 32/mm³. The blood leukocytes were demonstrated to be of donor origin by analysis of polymorphic enzyme markers, and 20 of 21 unstimulated marrow metaphases had the donor karyotype. This patient was treated conservatively with supportive care and recovered spontaneously. UPN 2151 developed progressive pancytopenia and marrow hypoplasia on day 189, associated with persistence of donor T cells in peripheral blood but reappearance of host cells in the marrow. This patient received unmodified marrow from the original donor after reconditioning with cyclophosphamide and antithymocyte globulin but died of Escherichia coli bacteremia and pulmonary aspergillosis five days later, before engraftment could occur.

Table 4. Patients Prepared With 12.0 Gy Fractionated Total-Body Irradiation

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>T Cells Detected</th>
<th>GVHD Grade</th>
<th>Marrow Function</th>
<th>Survival (days)</th>
<th>Current Status/Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>2090</td>
<td>CML-Chr</td>
<td>yes</td>
<td>II (38)</td>
<td>Good</td>
<td>314</td>
<td>Pneumocystis pneumonia, chronic GVHD</td>
</tr>
<tr>
<td>2123</td>
<td>CML-Chr</td>
<td>yes</td>
<td>0</td>
<td>Transient failure (148)</td>
<td>&gt;425</td>
<td>Well</td>
</tr>
<tr>
<td>2151</td>
<td>ANL-Rem</td>
<td>no</td>
<td>0</td>
<td>Failure (189)</td>
<td>224</td>
<td>2d transplant</td>
</tr>
<tr>
<td>2157</td>
<td>ANL-Rem</td>
<td>no</td>
<td>0</td>
<td>Failure (55)</td>
<td>206</td>
<td>2d transplant</td>
</tr>
<tr>
<td>2230</td>
<td>CML-Chr</td>
<td>yes</td>
<td>II (26)</td>
<td>Relapsed (264)</td>
<td>281</td>
<td>Leukemia</td>
</tr>
<tr>
<td>2248</td>
<td>CML-Chr</td>
<td>no</td>
<td>0</td>
<td>Failure (21)</td>
<td>37</td>
<td>2d transplant</td>
</tr>
<tr>
<td>2261</td>
<td>CML-Chr</td>
<td>no</td>
<td>I</td>
<td>Failure/relapse (244)</td>
<td>311</td>
<td>2d transplant</td>
</tr>
<tr>
<td>2299</td>
<td>Lymphoma</td>
<td>no</td>
<td>0</td>
<td>Good</td>
<td>58</td>
<td>CMV interstitial pneumonia</td>
</tr>
<tr>
<td>2307</td>
<td>CML-Chr</td>
<td>no</td>
<td>I</td>
<td>Failure/relapse (195)</td>
<td>270</td>
<td>2d transplant</td>
</tr>
<tr>
<td>2343</td>
<td>ANL-Rem</td>
<td>yes</td>
<td>I</td>
<td>Failure (69)</td>
<td>177</td>
<td>3d transplant</td>
</tr>
<tr>
<td>2350</td>
<td>CML-Chr</td>
<td>yes</td>
<td>I</td>
<td>Good</td>
<td>&gt;225</td>
<td>Well</td>
</tr>
</tbody>
</table>

T cells were detected by fluorescent staining of treated marrow cells after culture in PHA for seven days (see Materials and Methods). UPN 2230 had thrombocytopenia after day 150, possibly related to sulfamethoxazole. Patient refused marrow aspiration. In UPN 2261 and UPN 2307, Ph⁺-positive cells were present in the marrow at the time of graft failure. In UPN 2299 on day 52, cytogenetic studies showed that seven out of 17 PHA-stimulated blood cells and one out of 61 unstimulated marrow cells were of host origin. UPN, unique patient number; CML-Chr, chronic myelogenous leukemia, chronic phase; ANL-Rem, acute nonlymphoblastic leukemia in remission; CMV, cytomegalovirus.

*Transien myeloid maturation arrest.
Graft failure and cytogenetic relapse. Three other patients (UPN 2046, UPN 2261, and UPN 2307) developed graft failure that was associated with cytogenetic but not morphological evidence of leukemic relapse (Tables 4 and 5). UPN 2046 was originally diagnosed as having a myelodysplastic syndrome in 1974. After treatment with corticosteroids and androgens, he improved until 1983, when he developed acute myelomonocytic leukemia. After three unsuccessful attempts to induce remission, he was referred for allogeneic marrow transplantation. A bone marrow aspirate on day 56 after transplantation showed 90% cellularity with ten of ten marrow metaphases that were of donor origin. Mixed chimerism was observed in stimulated peripheral blood cells on days 63 and 82 with a total of only three of 29 metaphases that were of donor origin. On day 82 the bone marrow aspirate was 100% cellular but showed erythroid hypoplasia and mixed chimerism, with 11 of 21 metaphases that were of host origin having consistent abnormalities indicative of leukemic relapse. On day 90 the marrow aspirate on day 209 showed 10% cellularity with no morphological evidence of leukemic relapse. However, nine of 21 marrow metaphases were Ph' positive. This patient received unmodified marrow from the original donor after reconditioning with cyclophosphamide and dimethylbu-sulfan. Prompt engraftment was observed, but the patient died of veno-occlusive disease of the liver.

UPN 2261 did well until day 244, when pancytopenia was noted. A marrow aspirate showed 20% cellularity with no morphological evidence of leukemic relapse. Cytogenetic analysis was not performed on this marrow. A marrow aspirated ten days later showed 10% cellularity with six of seven metaphases that were Ph' positive. This patient received unmodified marrow from the original donor after reconditioning with cyclophosphamide and dimethylbursulfan. Prompt engraftment was observed, but the patient died of systemic aspergillosis.

Graft-versus-host disease. Three study patients had grade II GVHD (Tables 4 and 5), and each responded to treatment with either corticosteroids (UPN 2090 and UPN 2082) or antithymocyte globulin (UPN 2230). None of the study patients died of acute GVHD, although one patient (UPN 2090) died of pneumocystis pneumonia after chronic GVHD. Patients in the study group had a significantly decreased incidence and severity of acute GVHD compared with controls (Table 6). This analysis combines results for both study patient subgroups since the preparative regimen did not correlate with GVHD among the controls, in which day 90 were of donor origin. The patient later developed granulocytopenia and died of bacterial pneumonia on day 101.

UPN 2307 did well until day 134, when a decreased leukocyte count of 2,000/μL was noted. A bone marrow aspirate showed 80% to 90% cellularity with 20 of 20 metaphases that were Ph' negative. During the ensuing weeks the leukocyte count continued to decrease slowly, but the hematocrit and platelet count remained stable. On day 177 he developed T3 to T10 dermatomal herpes zoster and was treated with acyclovir. On day 195 a bone marrow aspirate showed 30% cellularity with severe myeloid hypoplasia and megakaryocytic hyperplasia. Subsequently, the platelet count decreased to less than 20,000/μL, and a marrow aspirate on day 209 showed 10% cellularity with no morphological evidence of leukemic relapse. However, nine of 21 marrow metaphases were Ph' positive. This patient received unmodified marrow from the original donor after reconditioning with cyclophosphamide and dimethylbursulfan. Prompt engraftment was observed, but the patient died of veno-occlusive disease of the liver.

Fig. 2. Cumulative probability of engraftment of granulocytes to >1,000/μL, lymphocytes to 200/μL, and leukocytes to 1,000/μL in study patients (—) and in controls (····). Engraftment was defined as the first day that the cell count exceeded the indicated level for three or more consecutive days. Two of the 65 external control patients were not included because of early death on days 11 and 19, respectively. The differences in engraftment of lymphocytes and leukocytes between the two groups were statistically significant (P < .01, Wilcoxon two-sample rank sum test).
The difference between the 19 study patients and the controls is statistically significant \( P < .004 \), Mann-Whitney U test.

*Analysis excludes the seven study patients who had irreversible graft failure. The difference between these 13 study patients and the controls is statistically significant \( P < .02 \), Mann-Whitney U test.

†Two of the 65 external control patients were not included because of early death without GVHD on days 11 and 19, respectively. More than 80% of the controls who developed grade II through IV acute GVHD had the onset of disease before day 26, and the latest onset occurred on day 42.

The incidence of acute GVHD was 50% in patients who received 12.0 Gy of total-body irradiation and 52% in patients who received 15.75 Gy of total-body irradiation. With Kaplan-Meier censoring for early death, the incidence of grades II through IV acute GVHD was 16% in the entire study group and 24% if the seven patients with irreversible graft failure are excluded, compared with 52% in the control group \( P = .006 \) and 0.07, respectively, generalized log rank test.

### Table 6. Acute Graft-Versus-Host Disease

<table>
<thead>
<tr>
<th>GVHD Grade</th>
<th>Study Patients* No. (%)</th>
<th>Study Patients† No. (%)</th>
<th>External Controls‡ No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10 (53)</td>
<td>7 (54)</td>
<td>16 (25)</td>
</tr>
<tr>
<td>I</td>
<td>6 (32)</td>
<td>3 (23)</td>
<td>15 (24)</td>
</tr>
<tr>
<td>II</td>
<td>3 (16)</td>
<td>3 (23)</td>
<td>10 (16)</td>
</tr>
<tr>
<td>III</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>17 (27)</td>
</tr>
<tr>
<td>IV</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>5 (8)</td>
</tr>
</tbody>
</table>

*Analysis excludes one study patient who had graft failure on day 21.

†Analysis excludes the seven study patients who had irreversible graft failure. The difference between these 13 study patients and the controls is statistically significant \( P < .004 \), Mann-Whitney U test.

‡Two of the 65 external control patients were not included because of early death without GVHD on days 11 and 19, respectively. More than 80% of the controls who developed grade II through IV acute GVHD had the onset of disease before day 26, and the latest onset occurred on day 42.

### Discussion

The feasibility of preventing acute GVHD by removing mature T cells from the marrow graft has been amply demonstrated in animal model systems.13-16 In mice, treatment of the graft with an anti-theta antiserum alone is sufficient to prevent GVHD.13 In a previous clinical trial, however, it was found that treatment of human donor marrow with monoclonal anti-T cell antibodies in the absence of exogenous complement did not prevent GVHD.22 This result may suggest that in vivo mechanisms were inadequate to eliminate the antibody-coated T cells in marrow transplant recipients. In the present study the same antibodies were used together with rabbit serum complement to achieve 2- to 3-log in vitro depletion of T cells in donor marrow. Preliminary studies had demonstrated that complement-mediated lysis of human T cells was more efficient with the use of multiple anti-T cell antibodies, that two cycles of treatment with rabbit serum complement were superior to a single cycle of treatment, and that 2- to 3-log lysis of T lymphocytes could be accomplished without excessive damage to committed hematopoietic precursors assayed as BFU-E and CFU-GM (unpublished observations). The single lot of rabbit serum and procedures used for the present study caused little, if any, toxicity to these cells (Table 3).

In this study, eight of the patients who received T cell-depleted marrow had graft failure. This was transient in one patient but apparently irreversible and a cause of death in the others. The reconditioning regimens of antithymocyte globulin and cyclophosphamide or dimethylbusulfan and cyclophosphamide administered in preparation for regrafting caused severe hepatic and renal toxicity in four of the six patients who had second transplants. In three of the eight patients with graft failure, there was cytogentic evidence of leukemic relapse at the time that graft failure was noted. It is possible that the recurrent leukemia was responsible for suppression of hematopoesis in these patients.26-29 More likely, leukemic cells present in small numbers merely became evident after the graft failed for other reasons.

Graft failure or rejection represents a highly unusual outcome after HLA-identical marrow transplantation in patients with hematologic malignancies.8 In other settings, particularly in patients with aplastic anemia and in animal models, a history of prior transfusions, the marrow cell dose, the pretransplant and posttransplant immunosuppressive regimens, and the degree of genetic disparity between the host and the recipient have been identified as factors affecting the risk of graft rejection.40-42 The graft failures in this study did not appear to be caused by albsensitization related to previous transfusions, since four of the eight patients had never been transfused. Among the 20 study patients, the degree of T cell depletion alone did not appear to correlate with graft failure because two of the eight patients who had difficulty with engraftment received marrow in which PHA-driven T cell blasts were detectable after treatment (Table 4). The donor marrows given to the eight patients with graft failure could not be distinguished from the others on the basis of the numbers of viable nucleated cells, BFU-E or CFU-GM (data not shown). However, these assays would not detect damage to pluripotent stem cells caused by rabbit complement, by heterophile antibodies in the rabbit serum, by one or more of the anti-T cell antibodies, or by the in vitro manipulation itself. On the other hand, seven of the cases of graft failure occurred among the eleven patients who received 12.0 Gy of total-body irradiation and only one occurred among the nine patients who received 15.75 Gy of total-body irradiation. The number of patients at risk for graft failure in the latter group was small, since three of the nine patients died of transplant-related causes before day 100 and four others developed leukemic relapse between 123 and 225 days after transplantation. Nonetheless, the association between graft failure and irradiation dose would suggest that the graft failures were not caused solely by in vitro treatment-related damage to hematopoietic stem cells and that host factors were involved.

Because graft failure seldom occurs in recipients of unmodified HLA-identical allogeneic marrow transplants, the results of this study suggest that certain T cells in the donor marrow may facilitate sustained engraftment after allogeneic marrow transplantation. This hypothesis is consistent with observations that thoracic duct lymphocytes can facilitate engraftment in dogs given marrow grafts from unrelated DLA-nonidentical donors,43 an effect that can be abrogated by administration of cyclosporine.44 Donor T cells could facilitate sustained engraftment by acting as primary helper cells that promote hematopoietic stem cell growth or by eliminating residual host cells that otherwise might react against donor antigens and cause graft failure. Our data...
suggest that a helper T cell effect is not required in order to restore hematopoiesis because initial engraftment was rapid even when the marrow contained less than 0.03% T cells. The association of graft failure with irradiation dose and the reappearance of host cells in the blood or marrow in seven of the patients with graft failure are consistent with the concept that T cells in the marrow graft may suppress or eliminate host effector cells that survive the preparative regimen. Irradiated recipients of unmodified marrow grafts are usually, but not always, reconstituted with cells that are completely of donor origin.4,45 The occurrence of graft failure late after transplantation suggests that donor stem cell-derived T cells newly generated within the host environment cannot substitute for the effect of mature T cells in the donor marrow.

The results of this study suggest that depleting T lymphocytes from donor marrow can reduce the incidence of acute GVHD after HLA-identical allogeneic bone marrow grafting. Patient age and diagnosis, donor sex-match, and protective isolation have been identified previously as factors predictive of the risk of GVHD.5,23,34,46 In this study the a priori selection criteria assured comparability for age and diagnosis between the study group and the external controls. Similar numbers of patients in both groups received protective isolation. A larger proportion of the study patients had sex-matched donors, but sex-matching did not correlate with GVHD among the controls, where the incidence of acute GVHD was 59% in patients with sex-matched donors and 49% in patients with sex-mismatched donors. The incidence of GVHD among the controls was also not influenced by the route of cyclosporine administration (data not shown). Thus, although the two groups in this study were not contemporaneous, they appeared to be comparable for factors known to affect the risk of acute GVHD. Therefore, the reduced incidence of acute GVHD in the study group was most likely due to the single experimental variable of in vitro marrow treatment.

Our conclusion that GVHD can be prevented by depletion of T cells in the donor marrow is in accord with recently published results46 of a similar clinical trial at the Royal Free Hospital (RFH) that also used monoclonal anti-T cell antibodies and rabbit serum complement for treatment of donor marrow. In the RFH trial, two cases of grade I GVHD were observed among 14 patients. The apparently higher incidence of acute GVHD in our study could be related to a number of differences between the two trials. First, the median age of the Seattle patients was 35 and all were over age 30 and thus had a high risk of GVHD, whereas the median age of the RFH patients was 18 and only three of the 14 were age 30 or older. Second, it is possible that T cells were less efficiently depleted with the method used in Seattle and that patients received larger numbers of viable T lymphocytes. In fact, two of the three cases of grade II GVHD in our study occurred among the five patients who had received donor marrow in which PHA-driven T cell blasts were detected after treatment (Table 4). Third, our patients received cyclosporine after transplantation, whereas the RFH patients received no posttransplant immunosuppression. Cyclosporine withdrawal can produce a GVHD-like syndrome in rats after syngeneic bone marrow transplantation.46 However, the GVHD in our patients was unrelated to changes in cyclosporine dose.

Graft failure was not noted in the RFH study, although engraftment of granulocytes was somewhat slower than expected, unlike our results. The higher incidence of graft failure in our study could be related to differences in the methods used to treat the marrow or differences in the pretransplant or posttransplant immunosuppressive regimens. The possibility that host T cells or hematopoietic cells reemerged in some of the RFH patients has not been formally disproved, since careful studies of hematopoietic chimerism after day 100 have not been reported. Our studies of chimerism were limited because most of the patients had sex-matched donors. Mixed chimerism without graft failure was documented in one patient and could have occurred in others.

The findings of this study demonstrate the feasibility of preventing acute GVHD after HLA-identical allogeneic bone marrow transplantation by removing mature T cells from the graft. They further emphasize the role played by T cells in helping to maintain sustained hematopoietic function in recipients of HLA-identical marrow grafts. We postulate that certain T cells in the donor marrow have an effect that prevents residual host cells from suppressing or rejecting donor hematopoietic stem cells. The low incidence of graft failure in patients who received 15.75 Gy fractionated total-body irradiation suggests that the host cells capable of causing graft failure may be only relatively radioresistant. It is also possible that these patients were more immunosuppressed before transplantation because of prior therapy or more advanced disease. Nonetheless, it appears that the optimal application of in vitro T cell depletion for preventing GVHD will require more effective immunosuppression of the recipient in order to assure sustained engraftment and function of donor hematopoietic stem cells. Alternatively, T cell subset depletion might be feasible if the antihost effect required for sustained engraftment can be produced by cells that do not cause GVHD.

Outcomes other than engraftment and acute GVHD were not formally compared in this study. Obvious differences between the groups in the incidence of interstitial pneumonia and chronic GVHD and the rate of leukemic relapse were not apparent. Because of the increased incidence of graft failure, T cell depletion of donor marrow was associated with decreased survival, especially in the patients who received 12.0 Gy of total-body irradiation. Randomized trials in homogeneous patient populations at low risk for graft failure will be necessary in order to confirm the effect of depletion of T cells in the donor marrow on acute GVHD and to assess whether such procedures can improve survival after allogeneic bone marrow transplantation.

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REFERENCES


42. UCLA Bone Marrow Transplant Team: Prevention of graft rejection following bone marrow transplantation. Blood 57:9, 1981


Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts

PJ Martin, JA Hansen, CD Buckner, JE Sanders, HJ Deeg, P Stewart, FR Appelbaum, R Clift, A Fefer and RP Witherspoon