CONCISE REPORT

Clonable T Lymphocytes in T Cell-Depleted Bone Marrow Transplants Correlate With Development of Graft-v-Host Disease


Early clinical trials using T lymphocyte-depleted human marrow for transplantation have reported that such grafts reduce, to varying degrees, both the incidence and the severity of graft-v-host disease (GVHD). However, to date, no clear estimates have been made as to what degree of T cell depletion is necessary to prevent GVHD in every case. To address this problem, we used a limiting dilution assay (LDA) to quantitate residual clonable T lymphocytes in human T cell-depleted bone marrow in 31 HLA-identical transplants for leukemia. The number of phytohemagglutinin -interleukin 2-responsive T lymphocytes determined by LDA and expressed as T cell per kilogram recipient weight was found to correlate with the subsequent development of GVHD: no patients who received less than 1 x 10^5 T cell per kilogram developed GVHD (N = 24). Of the seven patients who received 1 x 10^5 to 4.4 x 10^5 T cell per kilogram, four patients developed grade I or II skin GVHD. This study thus provides a quantitative estimate of the number of T lymphocytes necessary to initiate clinically detectable GVHD in an HLA-identical host.

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GRAFT-v-HOST DISEASE (GVHD) following a bone marrow transplant (BMT) from an HLA-identical donor occurs in 50% to 70% of leukemic transplant recipients and is sufficiently severe (grade II through IV) to warrant treatment in 30% to 55% of cases, depending on the selection of pretransplant and posttransplant immunosuppression. In several animal models it has been shown that removal of donor T lymphocytes from histoincompatible bone marrow or spleens can prevent GVHD. Concomitantly, addition of graded numbers of spleen cells or thymocytes to a marrow inoculum increases the incidence and severity of this reaction. In one murine model of marrow transplantation, minor alloantigenic differences between an H-2-compatible donor and recipient result in a high incidence of lethal GVHD: contamination of 10^2 donor marrow cells with as few as 0.3% T cells (3 x 10^7 T lymphocytes) is sufficient to cause lethal GVHD.

Preliminary clinical trials with bone marrow depleted of donor T lymphocytes indicate that both the incidence and the severity of GVHD in engrafted patients undergoing BMT for treatment of leukemia are greatly reduced. However, neither the degree of T cell depletion required for the prevention of GVHD nor the number of T cells required to produce GVHD in humans has been established.

To quantitate the number of T lymphocytes in a bone marrow sample, we have developed a limiting dilution assay (LDA) that can detect 1 x 10^5 T lymphocytes in a total of 1 x 10^6 progenitor-enriched bone marrow cells. In initial experiments, we demonstrated that a 3- to 4-log depletion of clonable T cells could be achieved by differential agglutination with soybean agglutinin (SBA), followed by depletion of cells forming rosettes with sheep red blood cells (E'). We have now prospectively studied 31 BMTs that had been depleted of T lymphocytes (SBA E'). The results of this analysis suggest that the number of residual clonable T lymphocytes correlates with the absence or occurrence of GVHD in leukemic patients who received HLA-identical T lymphocyte-depleted bone marrow.

MATERIALS AND METHODS

Thirty-one patients with leukemia received an HLA genotypically identical bone marrow that had been depleted of T lymphocytes. All patients were treated with protocols approved by the Internal Review Board at Memorial Sloan-Kettering Cancer Center, and informed consent was obtained before transplantation. The median age of the patients was 25 years (range, 1.5 to 42 years). The patients were prepared for transplant by cytoreduction with fractionated total body irradiation (13.2 to 14.4 Gy) and cyclophosphamide (120 mg/kg) as previously described. All patients except one were hospitalized in single-room isolation without laminar flow or skin and mucosal decontamination. All patients received bone marrow that had been depleted of T lymphocytes by soybean lectin agglutinin (SBA) and E rosette depletion (E'), according to a modification of the method of Reiner et al. By the LDA used for the present report, this technique reproducibly achieves a 3- to 4-log depletion of clonable T cells, while preserving hematopoietic progenitors. None of these patients received any immunosuppression as prophylaxis against GVHD after BMT. Engraftment was documented by standard cytogenetic analysis, using either sex chromatin or quinacrine banding characteristics to differentiate donor and host hematopoietic cells. The diagnosis of GVHD was established according to the clinical criteria of Glucksberg et al and the histological criteria of Slavin and Woodruff.

Each T cell-depleted marrow was sampled before transplantation and evaluated for residual T lymphocytes by enumeration of E rosettes and by a LDA technique that we have developed. This

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technique determines the frequency of T lymphocytes responsive to phytohemagglutinin (PHA) and interleukin 2 (IL 2) in a sample of $1 \times 10^4$ bone marrow cells. This assay, described previously, has been modified to increase the T cell cloning efficiency. Briefly, T cell-depleted bone marrow fractions that had been maintained for up to 18 hours at 4°C in medium-199 supplemented with DNAase 30 

\[ \mu/mL \] (Sigma Chemical Co, St Louis), 10% fetal bovine serum, penicillin 100 \[ \mu/mL \], and streptomycin 100 \[ \mu/mL \] were washed and resuspended with irradiated (4,000 rad) allogeneic peripheral blood mononuclear cells as feeder cells (0.5 \[ \times \] 10^6/mL) in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 15% pooled human serum, penicillin 100 \[ \mu/mL \], streptomycin 2 \[ \mu/mL \], PHA-16 4 \[ \mu/mL \], and a prescreened optimum concentration of IL 2 containing supernatant derived from a Gibbon cell line (MLA), as previously described.\(^{23}\) T cell-depleted marrow was seeded at 800, 600, 400, and 200 cells per well in 60-well Terasaki plates (Nunc, Thomas Scientific, Philadelphia). A total of 120 replicate samples per cell concentration were plated. After 11 days' incubation at 37°C in a humidified atmosphere containing 5% CO\(_2\) in air, the wells were scored as positive or negative for growth by microscopic examination. Because the relationship between the number of cells seeded per well and the fraction of negative wells at each concentration is a linear one, and thus consistent with single-hit kinetics, it is possible to establish an equation for the line of best fit using minimum chi-square statistics.\(^{23}\) The slope of the line reflects the frequency of clonable T lymphocytes. The absolute number of T cells in a bone marrow inoculum was calculated by multiplying the frequency of T lymphocytes \[ \times \] the total number of mononuclear cells in the marrow inoculum. The use of DNAase in the maintenance medium and PHA-16 rather than PHA-P as the mitogen has increased the efficiency of the cloning of T cells from unmodified marrow to 75% to 80% of the number of E rosette-positive cells in the untreated bone marrow. In previous experiments, cytofluorographic analysis of cells pooled from positive wells from T cell-depleted BM demonstrated 70% to 95% expression of the pan T cell surface antigen CD3.\(^{23}\)

The Kruskall-Wallis test was used to determine the statistical significance between the results obtained for each group of patients (GVHD \(!=\) no GVHD).

RESULTS

Of the 31 patients analyzed, each achieved durable engraftment of donor progenitor cells with full reconstitution of hematopoietic function. Of these patients, two developed grade I skin GVHD (12.5 and 25 years) and two developed grade II skin GVHD (1.5 and 5 years). Twenty-seven patients did not develop GVHD, and no patient developed gastrointestinal or hepatic GVHD. In this series, there was no correlation between the age of the recipient or sex of the donor and the development or severity of GVHD.

The results of LDA quantitating residual clonable T lymphocytes demonstrated a significant difference in the T lymphocyte frequency ($P < .016$) and in the absolute number of T lymphocytes per kilogram ($P < .001$) between those patients who did and did not develop GVHD (Table I). This difference is further demonstrated in Fig 1, in which the absolute number of residual clonable T lymphocytes determined by LDA and expressed as T cells per kilogram recipient body weight is plotted for each group of patients (panel A: no GVHD and panel B: GVHD). No patient who received less than $1 \times 10^5$ T cells per kilogram, four developed grade I or grade II skin GVHD. In the small sample of patients with GVHD, the dose of T cells (1.8 to 4.3 $\times 10^5$ T cells per kilogram) did not correlate with the severity of GVHD (I or II).

In contrast, the development of grade I or grade II skin GVHD did not correlate with the number of residual T lymphocytes in the T cell-depleted graft as measured by the percentage of E rosettes. In addition, neither the absolute number of T cells nor the number of T cells per kilogram calculated by the percentage of E rosette-positive cells distinguished patients who did or did not develop GVHD.

DISCUSSION

Investigators in several laboratories have quantitated residual T cells in T cell-depleted marrow grafts by enumerating E rosettes or cells reactive with T cell-specific monoclonal antibodies, or by assaying for cells responsive to in vitro stimulation with mitogen or allogeneic cells in an attempt to identify transplants at risk for GVHD. While each of these analyses have documented significant reductions in the number of T cells transplanted after T cell depletion, they have not provided any consistent evidence of a correlation between the number of residual T cells transplanted and the subsequent development or severity of GVHD.\(^{24}\) This may reflect the inability of these techniques to sensitively detect small numbers of T cells in large populations of actively proliferating marrow cells.

In our own initial analyses of a small series of SBA-"E" marrow grafts, we were not able to identify residual T cells by E rosette analysis, reactivity with T cell-specific monoclonal antibodies, or response to mitogens or allogeneic

Table 1. Quantitation of Residual T Lymphocytes in GVHD

<table>
<thead>
<tr>
<th></th>
<th>No GVHD (N = 27)</th>
<th>GVHD (N = 4)</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Limiting dilution analysis</td>
<td></td>
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<tr>
<td>Frequency ($10^{-4}$)</td>
<td>1.33 (0.16–5.5)</td>
<td>2.96 (1.84–7.4)</td>
<td>.016</td>
</tr>
<tr>
<td>T cells ($\times 10^4$)</td>
<td>1.93 (0.95–8.57)</td>
<td>6.78 (1.49–28.12)</td>
<td>.112</td>
</tr>
<tr>
<td>T cells per kilogram ($\times 10^4$)</td>
<td>3.72 (0.92–16)</td>
<td>23.99 (18.63–43.93)</td>
<td>.001</td>
</tr>
<tr>
<td>E rosettes</td>
<td></td>
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<tr>
<td>Percentage</td>
<td>0.33 (0.1–1.5)</td>
<td>0.4 (0.3–7)</td>
<td>.856</td>
</tr>
<tr>
<td>T cells ($\times 10^4$)</td>
<td>4.00 (0.58.3)</td>
<td>1.22 (0.111)</td>
<td>.430</td>
</tr>
<tr>
<td>T cells per kilogram ($\times 10^4$)</td>
<td>6.31 (0.88.1)</td>
<td>30.04 (0.278.0)</td>
<td>.154</td>
</tr>
</tbody>
</table>

Results are expressed by the median and the range. P value was determined by the Kruskall-Wallis test.
cyclosporine compared with cyclosporine acute graft versus host disease after marrow transplantation for cell cloning. The latter technique provides optimal condi- 
T, Sullivan K, Witherspoon R, Yee cells by monoclonal antibody analysis. Furthermore, T lymphocytes did not proliferate above 772 for selective for SBAE fraction, using conditions previously developed cultured in the presence of PHA and IL 2 for ten days. Transplant Proc in tetraparental bone marrow chimeras. marrow grafts J 4. von Boehm A, Sprent J, Nabholz M: Tolerance to histocom- 
patibility determinants in tetraparental bone marrow chimeras. J Exp Med 141:322, 1975 5. Muller-Rochholtz W, Wotzge H-U, Muller-Hermelink HK: Bone marrow transplantation in rats across strong histocompatibility barriers by selective elimination of lymphoid cells in donor marrow. Transplant Proc 8:537, 1976 were also unable to show a correlation between the number of residual T lymphocytes detected in the final fraction by E rosette formation and the subsequent development of GVHD. However, a clear and significant correlation between the number of clonable T lymphocytes in the SBA E marrow inoculum, and particularly the dose of T lymphocytes per kilogram body weight, and the subsequent development of GVHD was observed. Patients receiving \( \frac{1}{10^6} \) clonable T lymphocytes per kilogram did not develop GVHD, whereas four of seven patients receiving \( \frac{1}{10^7} \) T lymphocytes developed this complication.

The disparity in results obtained by these two methods of T cell quantitation may be due to any of several possibilities. Detection of E rosettes or T cells reactive with monoclonal antibodies depends not only on the concentration of T lymphocytes, but also on the density of the antigens or receptors on the surface of these cells. Small populations of T cells expressing these determinants at low density might not be differentiable from non-T cells in marrow fractions at the time of the T cell depletion but could be detected if cultured under conditions that have been shown to promote T cell growth and T cell antigen expression. We also cannot rule out the possibility that populations of allroreactive postthymic T cells capable of initiating GVHD exist that do not express these T cell determinants, but can be induced to express them under these culture conditions.

Nevertheless, the frequency of clonable T lymphocytes in this LDA correlates with the development of GVHD, and the present study demonstrates for the first time a direct correlation between T lymphocytes in the bone marrow graft and subsequent development of GVHD.

ACKNOWLEDGMENT

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Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease

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