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## Protective Conditioning for Acute Graft-versus-Host Disease

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### ABSTRACT

#### BACKGROUND

Conditioning with total lymphoid irradiation plus antithymocyte serum protects mice against acute graft-versus-host disease (GVHD) after hematopoietic-cell transplantation. We tested this strategy in humans.

#### METHODS

Thirty-seven patients with lymphoid malignant diseases or acute leukemia underwent an experimental conditioning regimen with 10 doses of total lymphoid irradiation (80 cGy each) plus antithymocyte globulin, followed by an infusion of HLA-matched peripheral-blood mononuclear cells from related or unrelated donors who received granulocyte colony-stimulating factor.

#### RESULTS

Of the 37 transplant recipients, only 2 had acute GVHD after hematopoietic-cell transplantation. Potent antitumor effects in patients with lymphoid malignant diseases were shown by the change from partial to complete remission. In the transplant recipients who underwent conditioning with total lymphoid irradiation and antithymocyte globulin, the fraction of donor CD4+ T cells that produced interleukin-4 after in vitro stimulation increased by a factor of five, and the proliferative response to alloantigens in vitro was reduced, as compared with normal control subjects and control subjects who underwent conditioning with a single dose of total-body irradiation (200 cGy).

#### CONCLUSIONS

A regimen of total lymphoid irradiation plus antithymocyte globulin decreases the incidence of acute GVHD and allows graft antitumor activity in patients with lymphoid malignant diseases or acute leukemia treated with hematopoietic-cell transplantation.

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**A**LLOGENEIC HEMATOPOIETIC-CELL transplantation with the use of conditioning regimens of nonmyeloablative radiotherapy, chemotherapy, or both to decrease early toxic effects extends the possibility of hematopoietic-cell transplantation to patients older than 50 years and those with coexisting conditions.<sup>1-4</sup> However, acute graft-versus-host disease (GVHD) remains a major problem after nonmyeloablative transplantation: acute GVHD (grade II or higher) developed in 20 to 65 percent of the patients in single-center or multicenter trials.<sup>5-10</sup> Death due to this complication accounts for approximately 50 percent of the deaths that are not due to a relapse of the neoplasm.<sup>3,4,10</sup>

A new approach to the prevention of acute GVHD takes advantage of the immune system's regulatory T cells. Two types of regulatory T cell in mice, natural killer T cells and CD4+CD25+ T cells, can prevent acute GVHD.<sup>11-14</sup> These regulatory T cells inhibit the proliferation of and cytokine secretion by CD4+ and CD8+ donor T cells that injure the intestines, liver, and skin in acute GVHD.<sup>11,14</sup> Nevertheless, direct tumor-killing activity mediated by donor CD8+ T cells remains unaffected.<sup>12</sup> Thus, regulatory T cells can separate GVHD from the antitumor activity of the graft. Regulatory natural killer T cells of either donor or host origin have the unique capacity to prevent acute GVHD by secreting interleukin-4.<sup>13-15</sup> Natural killer T cells recognize a nonpolymorphic, major-histocompatibility-complex class I-like antigen-presenting molecule, CD1d, which is present on both the donor and host antigen-presenting cells.<sup>16</sup>

Natural killer T cells constitute only 1 to 3 percent of all T cells in the spleen in normal mice. After repeated treatment with low-dose irradiation targeted to the spleen, thymus, and lymph nodes, however, the proportion of these cells progressively increases until ultimately they constitute the majority of T cells in the spleen and bone marrow.<sup>14,15</sup> This change probably results from the resistance of natural killer T cells, as compared with conventional T cells, to radiation-induced apoptosis due to increased expression of antiapoptotic genes by the natural killer T cells.<sup>17</sup> In preclinical studies, murine recipients of allogeneic bone marrow that underwent conditioning with anti-T-cell antibodies and repeated low-dose irradiation targeted to the lymphoid tissue (total lymphoid irradiation) were fully protected from GVHD, whereas mice that underwent conditioning with anti-T-cell antibod-

ies and a single dose of total-body irradiation were not protected.<sup>14,15</sup> Studies of the pattern of cytokine secretion by donor T cells in protected hosts showed a polarization toward a pattern of type 2 helper T (Th2) cells, with increased secretion of interleukin-4.<sup>15</sup> The Th2 cells assist B cells to produce antibodies and reduce the inflammation promoted by type 1 helper T cells.

In the present study, we adapted the conditioning regimen of total lymphoid irradiation that reduces the incidence of acute GVHD in rodents to the treatment of humans with lymphoid malignant diseases or acute leukemia.

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## METHODS

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### PATIENTS

Beginning on December 28, 2001, 37 consecutive patients were enrolled in a treatment protocol that was reviewed and approved by the Stanford University administrative panel on human subjects in medical research. Observations were carried out through December 1, 2004. Patients were eligible for the study if they had received a diagnosis of lymphoid malignant diseases or acute leukemia, were 50 years of age or older, were younger than 50 years but had preexisting medical conditions, or had received prior therapy and were considered to be at too high a risk for conventional myeloablative transplantation. For patients with lymphoid malignant diseases, no exclusions were made on the basis of disease status, sensitivity to chemotherapy, or prior bacterial or fungal infection. For patients with acute leukemia to be eligible, bone marrow aspirates were required to show less than 5 percent of blasts within six weeks before the start of the conditioning regimen. No patient refused to undergo the experimental conditioning regimen. All patients provided written informed consent. Patients were to be excluded if they were pregnant or if they had decompensated liver disease, a corrected pulmonary-diffusing capacity of less than 35 percent, a cardiac ejection fraction of less than 30 percent, a Karnofsky performance status of less than 50 percent, or serologic evidence of infection with the human immunodeficiency virus.

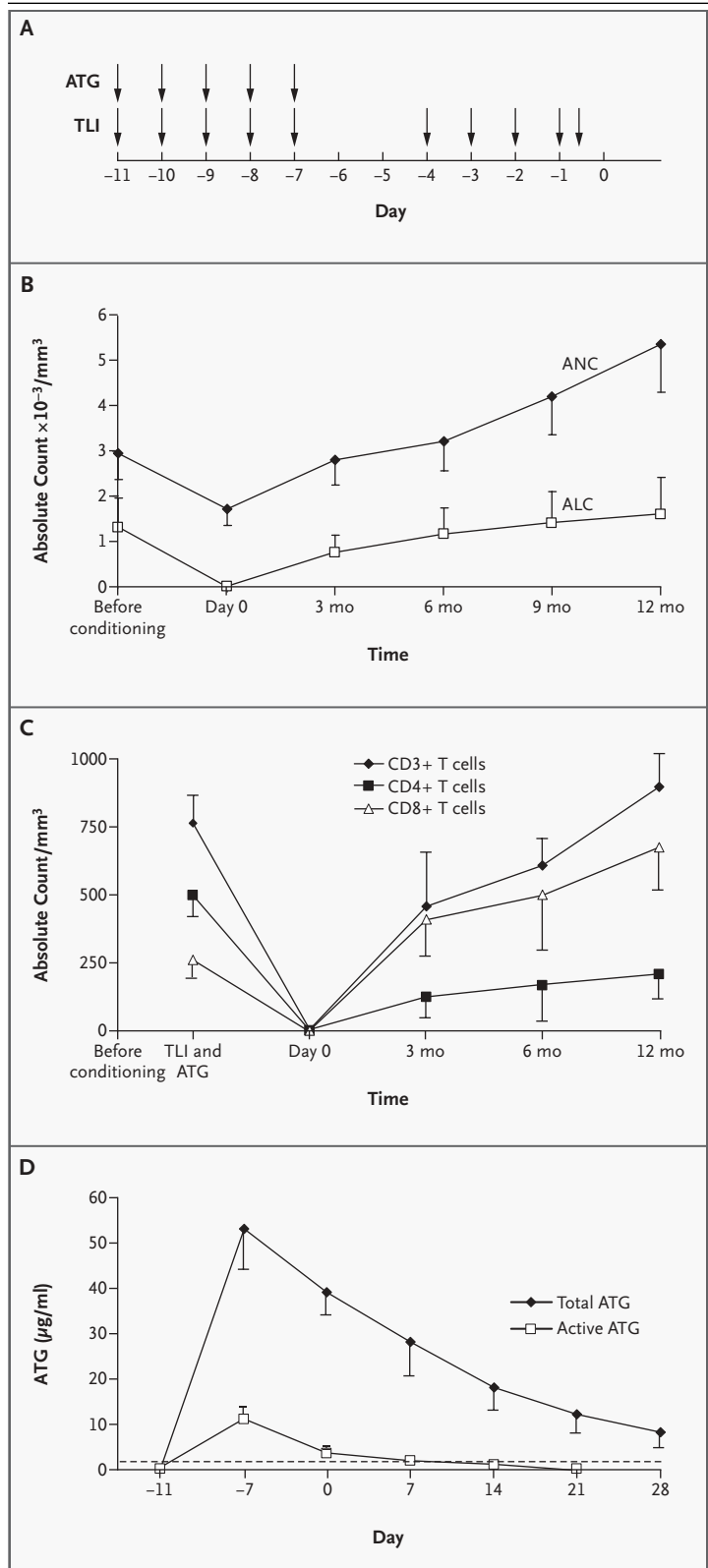
### THE REGIMEN

Total lymphoid irradiation was administered from a 15-MeV linear accelerator (photon beam) at a dose of 80 cGy daily, starting 11 days before transplantation, until a total of 10 doses (800 cGy) had been

**Figure 1. Nonmyeloablative Conditioning Regimen of Total Lymphoid Irradiation and Antithymocyte Globulin.**

Panel A shows the experimental conditioning regimen, which consisted of 10 doses of total lymphoid irradiation (TLI) (at 80 cGy each) given over a period of 11 days (as indicated by arrows). Antithymocyte globulin (ATG) (at a dose of 1.5 mg per kilogram of body weight per day) was administered intravenously on days -11 through -7 (as indicated by arrows). An intravenous infusion of mobilized HLA-matched peripheral-blood mononuclear cells from a donor was administered on day 0. Panel B shows the mean ( $\pm$ SD) absolute neutrophil count (ANC) and the mean absolute lymphocyte count (ALC) before the conditioning regimen was started, immediately after completion of the regimen (day 0), and at 3-month intervals after transplantation, up to 12 months. The data are from 18 consecutive patients who could be evaluated for all six points in time. The T bars indicate the standard deviation. Panel C shows the mean absolute counts of CD4+, CD8+, and CD3+ T cells before the conditioning regimen and after transplantation. The data are from 14 consecutive patients at each point in time. Assays were performed by the Stanford University Blood Bank Laboratories. Panel D shows the mean concentrations of total and active antithymocyte globulin in the serum before the conditioning regimen and after transplantation. Data are from 12 consecutive patients at each point in time. The dashed horizontal line represents the threshold for in vivo T-cell depletion.<sup>19</sup>

delivered. The irradiation consisted of a supradiaphragmatic mantle field, a subdiaphragmatic field that included an inverted Y, and splenic ports encompassing all major lymphoid organs, including the thymus, spleen, and lymph nodes, as used in the treatment of Hodgkin's disease.<sup>18</sup> Antithymocyte globulin (Thymoglobulin, SangStat), at a dose of 1.5 mg per kilogram of body weight per day, was given intravenously on days -11 through -7, with day 0 being the day of transplantation (Fig. 1A). All patients received prophylactic medications against bacterial, fungal, and viral infections and were monitored for cytomegalovirus and Epstein-Barr virus with the use of a blood polymerase-chain-reaction assay, as previously described.<sup>4,7</sup> Immunosuppressive therapy after transplantation included oral cyclosporine started on day -3, at a dose of 6.25 mg per kilogram twice per day, and mycophenolate mofetil, at a dose of 15 mg per kilogram twice a day, started on the first day after transplantation. Among recipients of matched grafts from related donors, cyclosporine was tapered to discontinuation from day 56 to day 180, and mycophenolate mofetil was stopped on day 28. Among recipients



of matched grafts from unrelated donors, cyclosporine was tapered to discontinuation from day 100 to day 180, and mycophenolate mofetil was tapered to discontinuation from day 42 to day 96.

#### MOLECULAR AND SEROLOGIC TYPING

Patients with sibling donors were serologically matched for HLA-A and B antigens and were matched by high-resolution DNA typing for HLA-DRB1 alleles. Patients with unrelated donors were matched for HLA-A, B, and DRB1 alleles with the use of high-resolution (DNA sequencing) molecular typing and for HLA-C and DQB1 alleles with the use of low-resolution molecular typing.

#### DONOR CELLS

Related and unrelated donors received a five-day course of subcutaneous granulocyte colony-stimulating factor (at a dose of 16  $\mu\text{g}$  and 10  $\mu\text{g}$  per kilogram of body weight per day, respectively), and mononuclear cells were harvested by leukapheresis. The CD34+ and CD3+ content of cells in the graft was determined according to the standard guidelines of the International Society of Hematology and Graft Engineering.<sup>20,21</sup>

#### ASSESSMENT OF CHIMERISM AND OF ANTITHYMOCYTE GLOBULIN IN SERUM

The status of hematopoietic chimerism was determined at intervals of one to six months after transplantation by DNA genotyping of simple sequence-length polymorphic markers that encode short tandem repeats, performed by the histocompatibility laboratory at the Stanford Medical School, as previously described in detail.<sup>22</sup> The analysis of chimerism was performed on whole blood and on blood mononuclear cells separated into T cells, B cells, and granulocytes with the use of immunomagnetic beads (Dyna) coated with monoclonal antibodies against CD3, CD19, and CD15, respectively. Samples were analyzed at SangStat for the presence of rabbit IgG, with the use of an enzyme-linked immunosorbent assay, and for the fraction of antithymocyte globulin that retained the capacity to bind to human CD3+ T cells (active antithymocyte globulin).<sup>19</sup>

#### CONTROL PATIENTS

We studied two groups of control patients. One group of four patients was treated for the myelodysplastic syndrome with hematopoietic-cell transplantation after undergoing conditioning with three

doses of fludarabine, at a dose of 30 mg per kilogram, followed by a single dose of total-body irradiation (200 cGy), and the same GVHD and antimicrobial prophylaxis as that received by patients who underwent the experimental conditioning regimen. The other group of five control patients received an allogeneic hematopoietic-cell transplant for the treatment of myeloma after undergoing conditioning with only total-body irradiation (200 cGy), as previously described.<sup>7,23</sup>

#### CYTOKINE SECRETION BY CD4+ AND CD8+ T CELLS

Peripheral-blood mononuclear cells from normal control subjects, control patients receiving total-body irradiation, and patients receiving total lymphoid irradiation were stimulated *in vitro* with phorbol myristate acetate and ionomycin for six hours, with brefeldin A (Sigma-Aldrich) added after two hours.<sup>12</sup> The cells were stained with allophycocyanin anti-CD4 monoclonal antibody and with phycoerythrin or fluorescein isothiocyanate anti-CD8 monoclonal antibody and were subsequently permeabilized with the use of a saponin-based reagent (Cytofix-Cytoperm kit, BD Bioscience). After permeabilization, the cells were stained for intracellular cytokines with the use of fluorescein isothiocyanate anti-interleukin-2 monoclonal antibody, phycoerythrin anti-interleukin-4 monoclonal antibody, fluorescein isothiocyanate anti-interferon- $\gamma$  monoclonal antibody, and fluorescein isothiocyanate anti-tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) monoclonal antibody, in accordance with the manufacturer's instructions. The cells were gated on CD4+ or CD8+ cells and analyzed for the percentage of gated cells that were positive on staining for each cytokine with the use of multicolor flow-cytometric analysis.<sup>12</sup>

#### MIXED-LYMPHOCYTE-REACTION ASSAY

Mononuclear cells from normal control subjects and from patients who had undergone total lymphoid irradiation or total-body irradiation were enriched for CD4+ T cells by incubation with phycoerythrin-anti-CD4 monoclonal antibody, with the use of antiphycoerythrin-conjugated immunomagnetic beads, and the cells were then purified on columns (MACS system, Miltenyi Biotech). The CD4+ cells were more than 90 percent pure, as judged by flow cytometry. These cells were tested for proliferative immune responses to alloantigens in culture for five days in complete medium with irradiated (5000 cGy) stimulator cells made from a pool of mononuclear cells obtained from three normal sub-

jects. The cultures were labeled with <sup>3</sup>H-thymidine during the last 20 hours and harvested, and the supernatants were assayed in a scintillation counter.<sup>24</sup>

**STATISTICAL ANALYSIS**

Comparisons of the values of the results of laboratory tests of samples obtained from patients in different groups and from normal control subjects were calculated with the use of the Wilcoxon two-sample rank test. Actuarial overall survival and progression-free survival were calculated with the use of a Kaplan–Meier analysis.

**RESULTS**

**PATIENTS' CHARACTERISTICS**

Table 1 summarizes the clinical features of the 37 patients enrolled in the study. Of these, 24 had lymphoid malignant diseases and 13 had acute leukemia. Twenty-eight patients had advanced disease, and 13 of the 24 patients with a lymphoid malignant disease had a relapse after undergoing autologous transplantation.

**CONDITIONING REGIMEN**

The total lymphoid irradiation regimen, consisting of 10 doses of lymphoid irradiation of 80 cGy each, was administered over a period of 11 days, and antithymocyte globulin was given on each of the first 5 days (Fig. 1A). Patients were admitted to the hospital for the administration of antithymocyte globulin. On completion of the regimen of total lymphoid irradiation, the patients were given an intravenous infusion of granulocyte colony-stimulating factor–mobilized mononuclear cells from HLA-matched related donors (23 patients, 62 percent) or unrelated donors (14 patients, 38 percent). The mean (±SD) absolute numbers of CD34+ hematopoietic progenitor cells and CD3+ T cells in these infusions were 5.8±2.4×10<sup>6</sup> per kilogram of body weight and 2.4±0.9×10<sup>8</sup> per kilogram, respectively.

We found that the nonmyeloablative regimen of total lymphoid irradiation and antithymocyte globulin caused only minor neutropenias (Fig. 1B). All the patients had persistent lymphopenia, with a marked reduction in CD4+ helper T cells, after transplantation (Fig. 1C). Despite the lymphopenia, antithymocyte globulin that bound to lymphocytes (as judged with the use of immunofluorescent staining) was not detected in the recipients' serum after day 7 (Fig. 1D). There were too few circulating host T cells at the time of transplantation to determine

**Table 1. Characteristics of Patients Undergoing Nonmyeloablative Conditioning with Total Lymphoid Irradiation (TLI) and Antithymocyte Globulin (ATG).\***

Characteristic	Value
No. of patients	37
Age — yr	
Median	52
Range	28–66
Diagnosis — no. (%)	
Acute myeloid leukemia, acute lymphoblastic leukemia, acute promyelocytic leukemia, or therapy-related acute myeloid leukemia	13 (35)
Mantle-cell lymphoma	9 (24)
Diffuse large-B-cell non-Hodgkin's lymphoma	5 (14)
Chronic lymphocytic leukemia or prolymphocytic leukemia	5 (14)
Follicular small-cleaved-cell non-Hodgkin's lymphoma	2 (5)
Hodgkin's disease	2 (5)
Peripheral T-cell lymphoma	1 (3)
Advanced disease — no. (%)†	28 (76)
No. of therapies before TLI and ATG	
Median	4
Range	2–9
Prior autologous transplantation — %	13 (35)
Donor — no. (%)	
Sibling	23 (62)
Unrelated	14 (38)
Sex mismatch — no. (%)	19 (51)
Cytomegalovirus serologic status — no. (%)	
Donor, recipient, or both seropositive	31 (84)
Donor and recipient seronegative	6 (16)
Disease status at time of TLI and ATG — no. (%)	
Complete remission	17 (46)
Partial remission	18 (49)
Progressive disease	2 (5)
Post-transplantation immunosuppressive therapy — no. (%)	
Cyclosporine or mycophenolate mofetil	37 (100)
Cell dose infused	
CD34+ ×10 <sup>-6</sup> /kg	5.8±2.4
CD3+ ×10 <sup>-8</sup> /kg	2.4±0.9

\* Plus–minus values are means ±SD.

† Advanced disease was considered to be acute leukemia with a high risk of cytogenetic abnormality or disease after the first remission (7 patients); or a lymphoproliferative disorder after the second complete remission, during partial remission, or with progressive disease at the time of total lymphoid irradiation and antithymocyte globulin (21 patients).

whether an increase in host natural killer T cells had occurred, as observed in the rodent spleen and bone marrow.<sup>14,15</sup>

Multilineage donor hematopoietic-cell engraftment, including T cells, B cells, and granulocytes,

was uniformly achieved within 56 days after transplantation (Fig. 2A). However, the donor T cells subsequently declined in number and were markedly reduced or undetectable within 75 to 200 days after transplantation in 6 of the 37 patients (Fig. 2B). The decline in the number of donor B cells and granulocytes was similar to that for the donor T cells (data

not shown). In four of these six patients, the loss of donor cells occurred during tumor progression or relapse.

**ACUTE GVHD**

All patients were monitored after transplantation for the principal manifestations of acute GVHD — diarrhea, rash, and abnormal results on liver-function tests. Standard scores for GVHD on a scale from grade 0 through grade IV were used during the first 100 days after transplantation to diagnose acute GVHD,<sup>25</sup> and thereafter, patients were evaluated for chronic GVHD, which was classified as absent, limited, or extensive.<sup>26,27</sup>

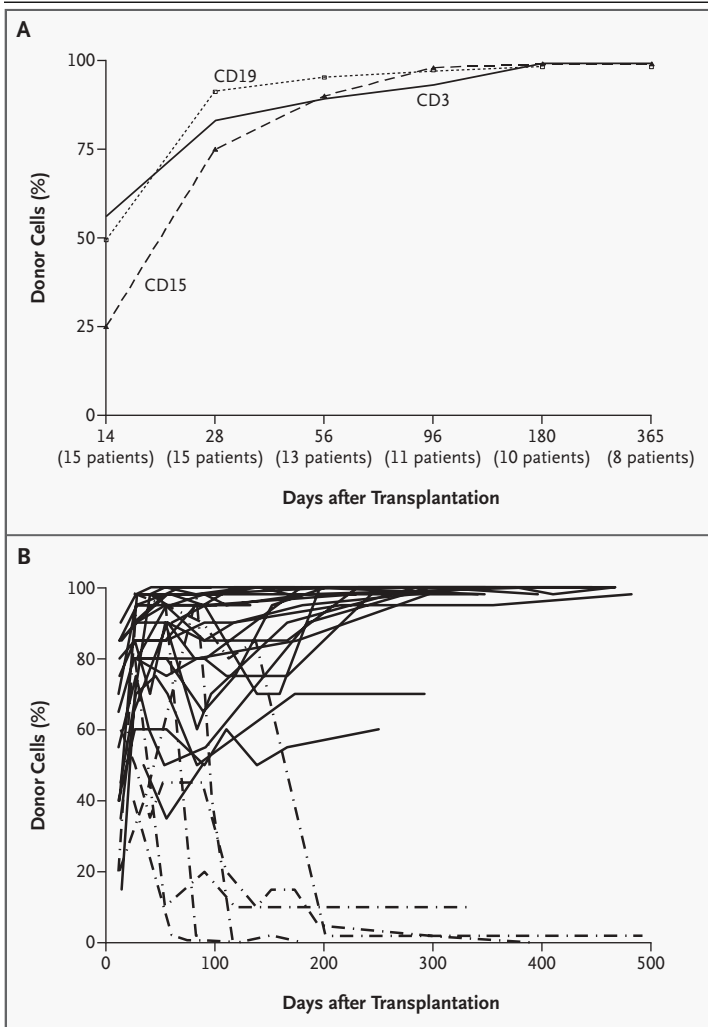
Among the 24 patients with lymphoid malignant diseases (of whom 13 received a transplant from a sibling and 11 from an unrelated donor), acute GVHD was scored as grade 0 in 22 patients, grade I in 1 patient, and grade III in 1 patient (Table 2). GVHD in this last patient responded to corticosteroids. Among the 13 patients with acute leukemia (of whom 10 received a graft from a sibling and 3 from an unrelated donor), acute GVHD was scored as grade 0 in all patients (Table 3).

Twenty patients with lymphoid malignant diseases survived for more than 100 days and were evaluated for the development of chronic GVHD. Among these patients, 14 had no evidence of chronic GVHD, 2 had limited chronic GVHD, and 4 had extensive chronic GVHD (Table 2). All 13 patients with acute leukemia survived for more than 100 days; 10 had no evidence of chronic GVHD, and 3 had extensive chronic GVHD (Table 3).

**SURVIVAL AND TUMOR RESPONSE**

Among transplant recipients with lymphoid malignant diseases, the follow-up for the first enrolled patient was 1069 days, and for the last enrolled patient it was 222 days; the median follow-up among the surviving patients was 482 days (Table 2). For the 13 patients with acute leukemia, the period of follow-up for the first patient enrolled and the last patient enrolled was 215 and 1041 days, respectively, and the median follow-up of surviving patients was 446 days (Table 3).

Of the 24 patients with lymphoid malignant diseases, 17 (71 percent) survived, all with a Karnofsky performance status score of 100 percent (indicating normal daily functioning) (Table 2).<sup>28</sup> Of the 13 patients with acute leukemia, 10 (77 percent) survived, all of them with a performance status of 90 percent or more (Table 3). For patients who died,



**Figure 2. Multilineage Chimerism in Patients Who Underwent Conditioning with Total Lymphoid Irradiation and Antithymocyte Globulin.**

Panel A shows the median percentages of donor-type cells among the CD3+ T cells, CD19+ B cells, and CD15+ neutrophils between 14 and 365 days after hematopoietic-cell transplantation in 15, 15, 13, 11, 10, and 8 consecutive patients at each of six points in time, respectively. Panel B shows the changes in the percentage of donor-type cells among CD3+ T cells in all 37 patients in the study. The lines represent all available data for each patient on the percentage of donor cells at each point in time up to 500 days, with solid lines representing patients with stable chimerism and dashed lines representing patients in whom chimerism was lost or markedly reduced.

**Table 2. Outcomes among the 24 Patients with Lymphoid Malignant Diseases.\***

Patient No.	Donor	Diagnosis	Acute GVHD (Grade)	Chronic GVHD (Extent)	Disease Status (At Entry/Current)	Survival Status (Days)	Current Performance Status (%)†
1	Related	MCL	0	Absent	PR/CR‡	Alive 1069	100
2	Unrelated	MCL	0	Extensive	PR/CR	Alive 1027	100
3	Related	DLCL-B	0	Limited	PR/CR‡	Alive 957	100
4	Related	FSCL	0	Absent	PR/CR	Alive 943	100
5	Unrelated	MCL	0	Absent	PR/—‡	Died 102	—
6	Unrelated	MCL	0	Extensive	PR/CR	Died 665	—
7	Unrelated	DLCL-B	III	Extensive	PR/PR‡	Died 171	—
8	Related	DLCL-B	0	Absent	PD/PD‡	Died 305	—
9	Unrelated	PLL	0	—	PD/PD	Died 99	—
10	Unrelated	MCL	0	—	PR/—	Died 44	—
11	Related	MCL	0	Absent	CR/CR	Alive 684	100
12	Unrelated	DLCL-B	0	Absent	PR/CR‡	Alive 656	100
13	Related	DLCL-B	0	Absent	PR/CR‡	Alive 614	100
14	Unrelated	MCL	0	Absent	CR/CR‡	Alive 614	100
15	Unrelated	CLL	0	Absent	PR/CR‡§	Alive 482	100
16	Related	CLL	0	Limited	PR/CR	Alive 411	100
17	Unrelated	PTCL	0	Not at risk¶	CR/Rel‡	Died 320	—
18	Related	CLL	0	Extensive	PR/CR	Alive 383	100
19	Unrelated	FSCL	I	Absent	PR/CR‡	Alive 377	100
20	Related	MCL	0	Absent	CR/CR§	Alive 369	100
21	Related	HD	0	Absent	PR/PD‡	Alive 362	100
22	Related	CLL	0	Not at risk¶	PR/PD	Alive 348	100
23	Related	MCL	0	Absent	PR/CR§	Alive 250	100
24	Related	HD	0	Absent	PR/PR‡	Alive 222	100

\* The cause of death of Patients 8 and 9 was progressive disease, of Patient 5 indwelling-line sepsis, of Patient 6 complications resulting from extensive chronic graft-versus-host disease (GVHD), of Patient 7 suicide, of Patient 17 recurrent lymphoma, and of Patient 10 thrombotic thrombocytopenia purpura. MCL denotes mantle-cell lymphoma; PR partial remission, CR complete remission; DLCL-B diffuse large-B-cell non-Hodgkin's lymphoma, FSCL follicular small-cleaved-cell non-Hodgkin's lymphoma; PLL prolymphocytic leukemia; PD progressive disease; CLL chronic lymphocytic leukemia; PTCL peripheral T-cell lymphoma; Rel clinical, radiologic, or histopathological relapse; and HD Hodgkin's disease. Dashes denote that the patient died before evaluation for chronic GVHD or tumor size (on computed tomography) or performance status.

† Performance status (as of December 1, 2004) was scored according to the Karnofsky scale, with higher scores indicating better daily functioning.<sup>28</sup>

‡ The patient had undergone a prior autologous transplantation.

§ The patient had tumor progression or a relapse after transplantation, was treated, and was in complete remission at the last follow-up visit.

¶ The patient was not at risk for chronic GVHD because of secondary graft loss at less than 100 days.

the causes of death are given in the footnotes to Tables 2 and 3.

Only 4 of the 24 patients who received a transplant as treatment for lymphoid malignant diseases were in complete remission when the regimen of total lymphoid irradiation and antithymocyte globulin was started. Eighteen of these 24 patients had evidence of residual disease (partial remission) on clinical examination or on computed tomogra-

phy, positron-emission tomography, or both, and two patients had progressive disease (Table 2). Of the four patients in complete remission at the time of transplantation, three were still in complete remission at the last follow-up visit and the fourth had a relapse. Of the 18 patients with measurable disease at the start of the conditioning regimen, 12 had complete remission without detectable acute GVHD. All patients whose condition changed from

**Table 3. Outcomes among the 13 Patients with Acute Leukemia.\***

Patient No.	Donor	Diagnosis	Chronic GVHD (Extent)	Disease Status (At Entry/Current)	Survival Status (Days)	Current Performance Status (%)†
25	Related	AML	Extensive	CR1/CR	Alive 1041	90
26	Related	AML	Extensive	CR1/CR‡	Alive 943	90
27	Related	AML	Extensive	CR1/CR	Alive 894	100
28	Related	AML	Absent	CR1/CR	Alive 496	100
29	Related	AML	Absent	CR2/CR	Alive 446	100
30	Related	AML	Absent	CR1/CR‡§	Alive 446	100
31	Unrelated	ALL	Absent	CR2/Rel	Died 232	—
32	Related	APL	Absent	CR4/Rel	Alive 425	90
33	Unrelated	AML	Absent	CR2/Rel‡	Died 214	—
34	Related	AML	Absent	CR1/CR	Alive 347	100
35	Related	AML	Absent	CR1/Rel	Died 220	—
36	Unrelated	tAML	Absent	CR1/CR‡	Alive 215	90
37	Related	AML	Absent	CR1/CR	Alive 215	100

\* No patients in this group had acute GVHD. Patients 31, 33, and 35 died as the result of a relapse. AML denotes acute myeloid leukemia; CR complete remission; CR1, CR2, and CR4 first, second, and fourth remission, respectively; ALL acute lymphoblastic leukemia; APL acute promyelocytic leukemia; Rel clinical, radiologic, or histopathological relapse; and tAML therapy-related acute myeloid leukemia. Dashes denote that the category is not applicable owing to the patient's death.

† Performance status (as of December 1, 2004) was scored according to the Karnofsky scale, with higher scores indicating better daily functioning.<sup>28</sup>

‡ The patient was at high risk or had complex findings on cytogenetic analysis.

§ The patient was treated for a relapse after transplantation and was in complete remission at the last follow-up visit.

partial to complete remission were in complete remission at the last follow-up visit. Four of 18 patients who had partial remission at the start of the conditioning regimen of total lymphoid irradiation and antithymocyte globulin still had evidence of disease at the last observation; of these 4 patients, 2 had disease progression. The two remaining patients who had partial remission at the time of transplantation died from causes other than relapse within six months after transplantation, and for that reason their disease status could not be ascertained by tumor imaging. However, autopsy revealed microscopical tumor foci in one patient (Patient 7).

All 13 patients with acute leukemia were in the first or a subsequent complete remission before starting the conditioning regimen. Remission status was assigned on the basis of histologic analysis of samples of bone marrow obtained six weeks before the start of the regimen. Of these 13 patients, 9 had a first complete remission and 4 had two or four remissions. Of the nine who had a first complete remission, seven continued to be in complete remission and two had a relapse. Of the four who

underwent transplantation while in a second or fourth complete remission, one continued to have a complete remission and three had a relapse. Among the patients with lymphoid malignant diseases, the actuarial overall survival and progression-free survival rates were 62 percent and 55 percent, respectively; among the patients with acute leukemia, the actuarial overall survival and progression-free survival rates were 73 percent and 69 percent, respectively.

#### SEVERE INFECTIONS

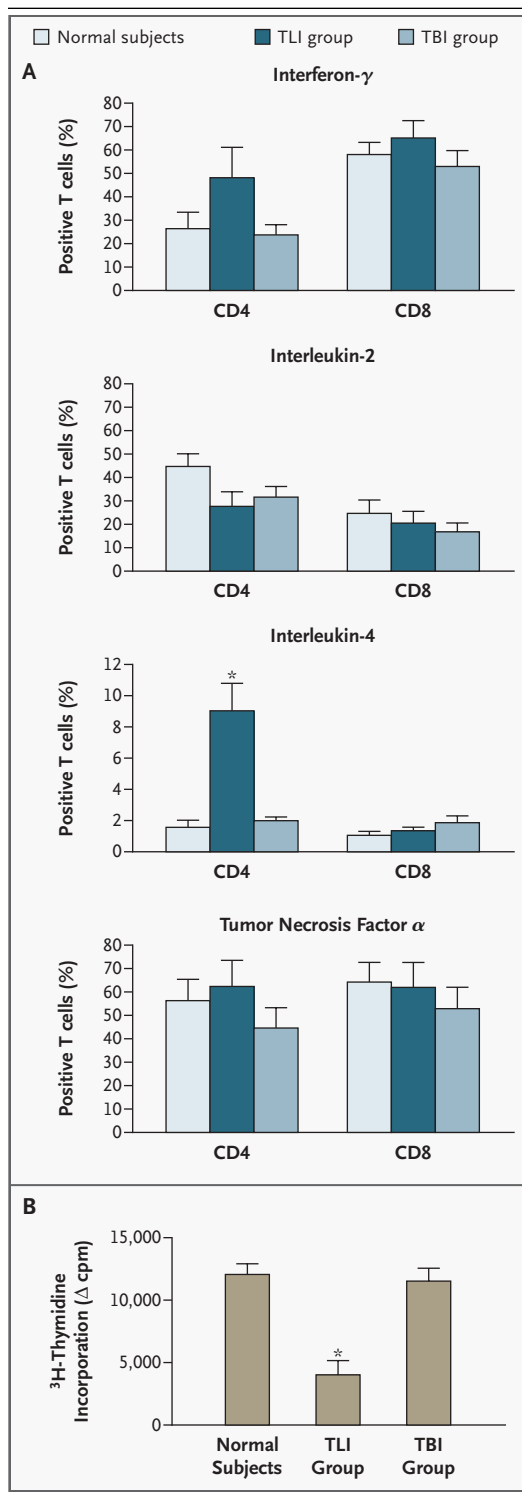
Four patients were hospitalized because of viral infection, fungal infection, or both: one patient had a gastrointestinal cytomegalovirus infection, one had a fungal pneumonitis, and two had multidermatomal herpes zoster or multifocal herpes simplex virus type 1 infection. These four patients and an additional five patients also had bacterial infections. All viral, bacterial, and fungal infections were controlled with the use of antimicrobial therapy, with the exception of one fatal case of bacteremia. In total, 28 of the 37 patients had no evidence of any clinically significant infection.

**Figure 3. Comparison of Intracellular Cytokine Production and the Proliferation of CD4+ and CD8+ T Cells.**

Panel A shows the percentage of CD4+ T cells and CD8+ T cells that stained positive for intracellular cytokines after in vitro activation by phorbol myristate acetate and ionomycin. T cells were obtained from patients who underwent total lymphoid irradiation (TLI) and from those who underwent total-body irradiation (TBI) and normal control subjects. Bars indicate the mean percentages of cytokine-positive cells, and T bars indicate the standard error in the groups of five to eight samples obtained from consecutive patients after TLI or TBI that had sufficient yields of CD4+ and CD8+ T cells for staining to be performed and in samples from normal controls. None of the patients had acute GVHD when the samples were drawn. Panel B shows proliferative responses of CD4+ cells, as measured by <sup>3</sup>H-thymidine incorporation after stimulation in the mixed-lymphocyte reaction. The mean <sup>3</sup>H-thymidine incorporation of triplicate assays for each patient was determined after subtracting the background value of the responder cells alone (Δ cpm). The mean values in five consecutive patients with sufficient yields of CD4+ T cells in each group are shown. The asterisks indicate a statistically significant difference (P≤0.05) between the TLI group and the other groups.

**FUNCTION OF CD4+ T CELLS**

We obtained purified mononuclear cells from the blood of the transplant recipients within one to seven months after transplantation and stimulated these cells in vitro with phorbol myristate acetate and ionomycin. The stimulated cells were analyzed for the accumulation in the CD4+ and CD8+ T cells of interferon-γ, interleukin-4, interleukin-2, and TNF-α with the use of immunofluorescent staining and flow cytometry. As shown in Figure 3A, the mean percentages of CD4+ and CD8+ cells that expressed intracellular interferon-γ, interleukin-2, or TNF-α did not differ significantly among the patients who had undergone total lymphoid irradiation, the normal controls, and the control patients who had received transplants and had undergone conditioning with low-dose total-body irradiation (P=0.13 to 0.94 for interferon-γ, P=0.06 to 0.90 for interleukin-2, and P=0.14 to 0.90 for TNF-α, as calculated with the Wilcoxon rank test). Control patients who had received transplants were those without acute GVHD. In contrast, among patients who had undergone total lymphoid irradiation, the mean percentage of CD4+ T cells that expressed interleukin-4 was significantly higher than among the normal controls (P=0.04 by the Wilcoxon rank test) or among control subjects who had undergone total-body irradiation with or without fludarabine



(P=0.05 by the Wilcoxon rank test); the mean percentage of interleukin-4-positive CD4+ cells in the total-lymphoid-irradiation group was about five times that in the control groups.

We compared the mixed-lymphocyte reaction of purified CD4+ T cells obtained from the normal controls and from the transplant recipients who had full chimerism and had undergone conditioning with either total lymphoid irradiation and antithymocyte globulin or low-dose total-body irradiation (Fig. 3B). Although there was no significant difference in the means between the group of normal controls and the group of patients who had undergone total-body irradiation ( $P=1.0$ ), the mean value for patients who had undergone total lymphoid irradiation was significantly reduced, as compared with normal controls and with patients who had undergone total-body irradiation ( $P=0.03$  for both comparisons by the Wilcoxon rank test).

#### DISCUSSION

In our study, the incidence of acute GVHD, grades II through IV, among recipients of hematopoietic-cell transplants who had undergone a conditioning regimen of total lymphoid irradiation plus antithymocyte globulin (1 of 37 patients, 3 percent) was markedly lower than that reported in studies of nonmyeloablative conditioning with total-body irradiation, chemotherapeutic agents, or both, conducted by researchers at our institution or elsewhere.<sup>1-10</sup> This reduction in the incidence of acute GVHD is especially noteworthy because 14 of the 37 patients received transplants from unrelated donors. In previous studies of recipients of hematopoietic-cell transplants from unrelated donors, the incidence of acute GVHD grades II through IV was more than 50 percent.<sup>5-7,9,10</sup>

In addition to the low incidence of acute GVHD among the transplant patients, 12 of 16 patients with lymphoid malignant diseases who entered the study while in partial remission and who were evaluated for tumor status after transplantation were in complete remission. The contributions of the conditioning regimen of total lymphoid irradiation and of the antitumor activity of the graft to these changes are difficult to distinguish from each other, except in seven patients who had clearing of the tumor outside the field of the total lymphoid irradiation. Of the 17 patients who entered the study in complete remission, 12 continued to have complete remission, with a median follow-up of 425 days.

In preclinical studies, the protection afforded against GVHD by the conditioning regimen of total lymphoid irradiation plus antithymocyte globulin in wild-type host mice was dependent on host

natural killer T cells, since protection was lost in mice in which the CD1d gene was inactivated, causing developmental failure of natural killer T cells in the thymus and other lymphoid tissue.<sup>15</sup> After the wild-type mice were treated with total lymphoid irradiation, the natural killer T cells were the main source of host interleukin-4, which subsequently increased the production of interleukin-4 by donor T cells.<sup>14,15</sup> In the current study, we were unable to measure directly the number and function of host natural killer T cells, because assays were limited to the blood samples obtained immediately after the patients underwent the conditioning regimen, and these samples contained too few host T cells for testing. Subsequently, we enrolled seven additional patients, and by increasing the volume of the blood samples obtained from each of the patients, we were able to measure the percentage of natural killer T cells in five of these patients. The median percentage of natural killer T cells among all T cells was increased by a factor of 10 after the patients underwent the experimental conditioning regimen, owing to a more profound decrease in the absolute number of non-natural killer T cells than of natural killer T cells (data not shown).

After the patients underwent transplantation, we found a marked increase in the production of interleukin-4 by donor CD4+ T cells in the transplant recipients, as compared with interleukin-4 production by CD4+ T cells in the normal control subjects, as was observed in mice.<sup>15</sup> These donor CD4+ T cells also showed a marked reduction in their proliferative response to alloantigenic stimulation in the mixed-lymphocyte reaction. Donor CD4+ T cells from patients who underwent conditioning with total-body irradiation, rather than total lymphoid irradiation plus antithymocyte globulin, showed neither an increased production of interleukin-4 nor a reduced proliferative response.

Although we could not distinguish the separate contributions of total lymphoid irradiation and antithymocyte globulin to protection against GVHD, preclinical studies have shown that the combination of total-body irradiation and antithymocyte globulin fails to provide protection against GVHD.<sup>14,15</sup> It is likely that the changes in the function of donor T cells in the patients who underwent total lymphoid irradiation reduced the risk of acute GVHD, since donor T cells with a profile of increased Th2 cytokine secretion have a decreased capacity to induce GVHD in rodents.<sup>29-31</sup>

In conclusion, a regimen of nonmyeloablative

conditioning of total lymphoid irradiation plus antithymocyte globulin given before hematopoietic-cell transplantation for lymphoid malignant diseases or acute leukemia can markedly decrease the incidence of acute GVHD while retaining the antitumor effect of the graft.

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**CORRECTION**

**Protective Conditioning for Acute Graft-versus-Host Disease**

Protective Conditioning for Acute Graft-versus-Host Disease . On page 1324, in the right-hand column, lines 1 and 2 should have read, "fludarabine, at a dose of 30 mg per square meter of body-surface area," rather than "30 mg per kilogram," as printed. Also, on page 1326, in the right-hand column, under Survival and Tumor Response, lines 6 through 9 should have read, "the period of follow-up for the first patient enrolled and the last patient enrolled was 1041 and 215 days, respectively," rather than "215 and 1041 days, respectively," as printed.