

TRANSPLANTATION OF ANERGIC HISTOINCOMPATIBLE BONE MARROW ALLOGRAFTS

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ABSTRACT

Background Successful allogeneic bone marrow transplantation relies on global immunosuppression or elimination of T cells. In contrast, the induction of anergy can inactivate specific sets of alloreactive T cells in the donor marrow. Previous work has shown that anergy can be induced by blocking the interaction of the B7 molecule on the surface of antigen-presenting cells with the CD28 molecule on the surface of T cells, thus preventing key signaling events essential for the activation of T cells. To investigate the feasibility of this approach with respect to transplantation of histoincompatible bone marrow, we undertook a clinical trial of ex vivo induction of anergy in T cells present in donor marrow to recipient alloantigens.

Methods Outcomes in 12 transplant recipients were evaluated. The recipients' peripheral-blood lymphocytes were collected before myeloablation and served as alloantigen-presenting cells. To induce alloantigen-specific anergy, bone marrow from a donor mismatched with the recipient for one HLA haplotype was cocultured with irradiated cells from the recipient for 36 hours in the presence of CTLA-4-Ig, an agent that inhibits B7:CD28-mediated costimulation. After conventional myeloablation and immunoprophylaxis, the treated donor cells were transfused into the recipient.

Results After the induction of anergy, the frequency of T cells capable of recognizing alloantigens of the recipient in donor marrow was sharply reduced ($P < 0.001$), whereas the responsiveness to alloantigens from persons unrelated to the recipient or the donor was unaffected ($P = 0.51$). In the 11 patients who could be evaluated, the haploidentical bone marrow cells engrafted. Of these 11 patients, 3 had acute graft-versus-host disease (GVHD) confined to the gastrointestinal tract. No deaths were attributable to GVHD. Five of the 12 patients were alive and in remission 4.5 to 29 months after transplantation.

Conclusions Donor bone marrow treated ex vivo to induce anergy to alloantigens from the recipient can reconstitute hematopoiesis in vivo with a relatively low risk of GVHD. (N Engl J Med 1999;340:1704-14.)

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Successful transplantation of organs requires the use of agents capable of suppressing the immune response against alloantigens.¹⁻³ Use of these nonspecific immunosuppressive drugs, however, can lead to the development of opportunistic infection or secondary cancer.⁴⁻¹³ For this reason, a way of specifically suppressing alloreactive T cells without inhibiting the entire T-cell repertoire is an important goal of transplantation immunology. One approach depends on the fact that the immune activation of T cells requires two signals from antigen-presenting cells.¹⁴⁻¹⁶ One of these signals is delivered to the T-cell receptor by an immunogenic peptide displayed in the context of the major histocompatibility complex. The other, a "costimulatory" signal, is generated by the interaction of certain cell-surface proteins on antigen-presenting cells and T cells. Without a costimulatory signal, signaling by the peptide T-cell-receptor complex does not result in T-cell immunity, but rather in a state of anergy, in which T cells are rendered specifically incapable of responding to the antigen that was presented to them in the absence of a costimulatory signal.¹⁶

A critical costimulatory signal is generated by the interaction between members of the B7 family of proteins on antigen-presenting cells and the CD28 molecule on the surface of T cells.¹⁷⁻¹⁹ Blockade of this interaction results in the induction of anergy.²⁰ Studies in animals have shown that interference with the B7:CD28 interaction permits successful transplantation of histoincompatible and even xenogeneic allografts without the need for pharmacologic immunosuppression.²¹⁻²⁴

In the case of bone marrow transplantation, T cells in the donor marrow are the cause of graft-versus-host disease (GVHD).²⁵⁻³⁰ We hypothesized that the induction of anergy in donor T cells that have the potential to react against the recipient's alloantigens might ameliorate GVHD while preserving the rest of the T-cell repertoire. One way to induce anergy in alloreactive T cells is by using a soluble prepara-

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TABLE 1. DEMOGRAPHIC CHARACTERISTICS AND DISEASE STATUS OF THE PATIENTS.*

PATIENT NO.	AGE (YR)/SEX	DISEASE	RESPONSE TO PRIMARY THERAPY†	NO. OF RELAPSES	PREVIOUS AUTOLOGOUS TRANSPLANTATION	STATUS AT TIME OF HSCT
1	4/F	B-cell acute lymphoblastic leukemia	Complete remission (16 mo)	1	No	Second complete remission
2	26/M	Diffuse large-cell lymphoma	Complete remission (29 mo)	2	Yes	Persistent disease
3	6/M	T-cell acute lymphoblastic leukemia	Induction failure followed by complete remission (7 mo)	1	No	Second complete remission
4	15/M	B-cell acute lymphoblastic leukemia	Induction failure; 4 regimens failed	NA	No	Persistent disease
5	7/F	Acute myelogenous leukemia	Induction failure; 2 regimens failed	NA	No	Persistent disease
6	23/M	T-cell lymphoblastic lymphoma	Complete remission (20 mo)	1	Yes	Persistent disease
7	7/F	B-cell acute lymphoblastic leukemia	Complete remission (35 mo)	1‡	No	Second complete remission
8	20/F	T-cell acute lymphoblastic leukemia	Complete remission (18 mo)	2	No	Third complete remission
9	12/M	Acute myelogenous leukemia	Induction failure; 3 regimens failed	NA	No	Persistent disease
10	1/F	Amegakaryocytic thrombocytopenia	NA	NA	NA	NA
11	0.5/F	Acute myelogenous leukemia	Induction failure; 3 regimens failed	NA	No	Persistent disease
12	16/M	T-cell acute lymphoblastic leukemia	Induction failure; 3 regimens failed	NA	No	Persistent disease

*HSCT denotes hematopoietic stem-cell transplantation, and NA not applicable.

†The duration of the first remission is shown in parentheses.

‡The initial attempt at reinduction therapy failed.

tion of CTLA-4, a counterreceptor for B7 that is found on activated T cells,³¹ to block the interaction between B7 proteins and CD28. As compared with CD28, CTLA-4 has a much higher affinity for B7, and thus it is a highly effective inhibitor of the B7:CD28 interaction.

We have previously shown that CTLA-4-Ig, a soluble fusion protein consisting of the extracellular domain of CTLA-4 linked to the constant region of IgG1, can reduce the alloreactivity of bone marrow T cells in vitro.³² We conducted a clinical trial of the feasibility of inducing anergy before the transplantation of histoincompatible bone marrow. We first incubated donor marrow cells with CTLA-4-Ig in the presence of mononuclear cells from the recipient. We then transferred the treated donor cells to the recipients and evaluated the outcomes in terms of the restoration of hematopoiesis and the incidence of GVHD.

METHODS

Patients

Patients were eligible for this study if they were ineligible for autologous stem-cell transplantation at our institutions on the basis of disease status and if they had no phenotypically or genotypically HLA-matched or single-antigen-mismatched family member who was available to serve as a donor, nor was there a phe-

notypically matched unrelated donor. Those likely to have rapid progression of disease during a search for an unrelated donor and those for whom a search was unsuccessful after four or more months were also eligible. Initially, patients were eligible if they had acute myelogenous or lymphoblastic leukemia, non-Hodgkin's or Hodgkin's lymphoma, multiple myeloma, or chronic lymphocytic leukemia that met prescribed criteria for high risk (those with advanced disease, those in relapse, or those with other poor prognostic features). Those with myelodysplasia or bone marrow failure became eligible for the study subsequently. All the patients had to be free of clinically significant organ dysfunction or active infection and had to have a life expectancy of at least six weeks. The institutional review boards of Children's Hospital and Dana-Farber Partners Cancer Care approved the protocol. Each patient or a legal guardian or parent gave written informed consent.

Between March 1996 and October 1998, 12 patients entered this study and underwent transplantation with haploidentical bone marrow (i.e., marrow from a donor who shared only one of two major-histocompatibility-complex haplotypes with the recipient) (Table 1). Follow-up is reported through February 20, 1999. The median age of the patients was 10 years. Eleven patients had hematologic cancer, and one had congenital amegakaryocytic thrombocytopenia. Of the 11 patients with hematologic cancer, 5 had never had a remission despite undergoing multiple treatments, 1 had not initially entered remission but did so subsequently, and 5 had entered a first remission readily. Only four patients underwent bone marrow transplantation while they were in remission. Of the remainder, most had virtually complete replacement of their marrow by malignant cells. Autologous transplantation had failed 27 and 11 months previously in Patients 2 and 6, respectively. The patients with acute leukemia in second remission, lymphoma in remission, bone marrow failure, or myelodysplasia without excess

TABLE 2. CHARACTERISTICS OF PATIENTS AND DONORS.

PATIENT NO.	PATIENT		DONOR		HLA TYPE*	
	AGE (YR)/SEX	AGE (YR)/RELATIONSHIP	NO. OF MISMATCHED ANTIGENS (HvG/GVHD)†	DONOR	RECIPIENT	
1	4/F	32/Mother	4/4	A32,B7,DR1303,DQ0301 A11,B55,DR1501,DQ0602	A32,B7,DR1303,DQ0301 A26,B27,DR0301,DQ02	
2	26/M	34/Half brother	2/1	A25,B18,DR1101,DQ0301 A0201,B7,DR1104,DQ0301	A25,B18,DR1101,DQ0301 A0201,B27,DR1101,DQ0301	
3	6/M	43/Father	4/4	A2,B37,DR4,DQ03 A28,B14,DR12,DQ03	A2,B37,DR4,DQ03 A24,B27,DR6,DQ01	
4	15/M	18/Sister	4/4	A30,B45,DR13,DQ0501 A1,B44,DR07,DQ02	A30,B45,DR13,DQ0501 A36,B53,DR03,DQ0402	
5	7/F	31/Father	4/4	A28,B57,DR0302,DQ0402 A30,B44,DR07,DQ02	A28,B57,DR0302,DQ0402 A57,B38,DR16,DQ0502	
6	23/M	44/Mother	4/4	A23,B70,DR0102,DQ0501 A30,B42,DR04,DQ0302	A23,B70,DR0102,DQ0501 A2,B7,DR13,DQ0604	
7	7/F	28/Mother	3/3	A0201,B27,DR1601,DQ0502 A0101,B8,DR03,DQ02	A0201,B27,DR1601,DQ0502 A0101,B57,DR1103/04/06, DQ0301	
8	20/F	50/Father	3/3	A68,B51,DR1501,DQ0602 A31,B7,DR0901,DQ02	A68,B51,DR1501,DQ0602 A31,B35,DR0807/0811,DQ0402	
9	12/M	53/Father	4/4	A66,B41,DR0801,DQ0402 A2,B41,DR07,DQ02	A66,B41,DR0801,DQ0402 A23,B49,DR0101,DQ0501	
10	1/F	3/Sister	2/2	A30,B18,DR0901,DQ02 A28,B35,DR0101,DQ0501	A30,B18,DR0901,DQ02 A25,B44,DR0101,DQ0501	
11	0.5/F	29/Father	3/4	A11,B52,DR0404/23,DQ0305 A24,B40,DR0404/23, DQ0303/06	A11,B52,DR0404/23,DQ0305 A3,B7,DR12,DQ0301	
12	16/M	37/Father	4/3	A26,B62,DR1101,DQ0301 A28,B27,DR0801,DQ0402	A26,B62,DR1101,DQ0301 A26,B44,DR1001,DQ0501	

*HLA class I typing was performed by serologic methods, and HLA class II typing was performed by polymerase chain reaction (PCR) with the use of sequence-specific oligonucleotide probes on all samples. In cases in which serologic methods were insufficient to resolve the specificity or in which there was potential matching between the donor and the recipient, class I typing was also performed by PCR with sequence-specific primers. In the case of Patient 8, molecular typing was not performed to resolve the phenotypic match at A31, because there was no documented heterogeneity of this allele. In the case of the donor for Patient 11, the use of PCR methods with sequence-specific oligonucleotide probes was unable to resolve whether the DR04 was homozygous (0404/0404) or heterozygous (0404/04023). The degree of mismatching for this pair was calculated with the assumption of homozygosity but may be greater if there was heterozygosity.

†HvG denotes host-versus-graft response (graft rejection), and GVHD graft-versus-host disease.

blasts were considered "good-risk patients," whereas all the others were considered "poor-risk patients."

Haploidentical relatives, including parents (nine cases), full siblings (two cases), and half siblings (one case), served as donors (Table 2). At the unshared haplotype, three donor-recipient pairs were matched at the A locus, one was matched at DR/DQ, and the remainder had no demonstrable phenotypic or genotypic match (Table 2). In eight cases both the recipient and the donor were seronegative for cytomegalovirus, in two cases both were seropositive, and in the remaining two cases the donor was seronegative and the recipient seropositive. All donors and recipients were seropositive for the Epstein-Barr virus except Patient 10 and her donor; the donor for Patient 2 was untested.

Treatment

The patients underwent leukapheresis to collect 200 million to 600 million mononuclear cells per kilogram of body weight for use as the recipient's alloantigen-presenting cells. These cells were cryopreserved with the use of a standard method. Subsequently, the patient received 1400 cGy of total-body irradiation³³ followed by cytarabine, cyclophosphamide, and methylprednisolone. Because of the high rate of toxic effects caused by this regimen in the first eight patients, cytarabine was not given to the last four patients.

Donor marrow was harvested two days before transplantation. Prophylaxis against GVHD consisted of short-course methotrexate and cyclosporine³⁴ starting on the day before transplantation, either by continuous infusion at 0.1 mg per kilogram per hour (in seven patients) or by a bolus of 1.5 mg per kilogram over a period of 2 to 3 hours every 12 hours (in five patients). The dose of cyclosporine was modified to alleviate toxic effects and was given orally as soon as gut function permitted. Methotrexate was withheld in cases of mucositis or renal insufficiency. Because of the high rate of regimen-related toxic effects in the first eight patients, the final dose of methotrexate was reduced and the administration of leucovorin was instituted for the last four patients (Table 3). GVHD was assessed with standard clinical criteria and graded according to the International Bone Marrow Transplant Registry (IBMTR) Severity Index.³⁵

The patients received oral, nonabsorbable antibiotics from the time of admission through the time of engraftment. All the patients received prophylaxis against fungal infection with fluconazole and against pneumocystis during conditioning and beginning again on day 30. The patients who were seropositive for herpes simplex virus received prophylaxis with acyclovir beginning five days before transplantation and continuing through day 24. If the donor or patient was seropositive for cytomegalovirus, high-dose acyclovir was administered.³⁶

TABLE 3. CLINICAL OUTCOMES OF PATIENTS ACCORDING TO DISEASE RISK.*

CATEGORY OF RISK/ PATIENT No.†	AGE (YR)/ SEX	DISEASE	DOSE OF METHOTREXATE (mg/m ²)				TIME TO ENGRAFTMENT (DAYS AFTER HSCT)‡	GRADE OF ACUTE GVHD/DATE OF ONSET§	STATUS	CAUSE OF DEATH
			DAY 1	DAY 3	DAY 6	DAY 11				
Good risk										
1	4/F	B-cell acute lymphoblastic leukemia	15	10	10¶	5¶	21	0	Died (day 22)	Diffuse alveolar hemorrhage
3	6/M	T-cell acute lymphoblastic leukemia	15	5¶	5¶	0	20	B/day 56	Alive, in complete remission (day 863)	
7	7/F	B-cell acute lymphoblastic leukemia	15	10	5¶	0	23	0	Alive, in complete remission (day 521)	
10	1/F	Amegakaryocytic thrombocytopenia	15¶	10¶	10¶	5¶	18	0	Alive, in complete remission (day 307)	
Poor risk										
2	26/M	Diffuse large-cell lymphoma	15	10¶	10¶	NE	NE	NE	Died (day 8)	Multiorgan failure and sepsis
4	15/M	B-cell acute lymphoblastic leukemia	15	5	0	0	Graft failure	0	Died (day 102)	Disseminated toxoplasmosis
5	7/F	Acute myelogenous leukemia	15¶	5¶	5¶	0	20	0	Died (day 39)	Aspergillus infection and multiorgan failure
6	23/M	T-cell lymphoblastic lymphoma	15	10¶	5¶	0	17	C/day 20	Died (day 31)	Multiorgan failure
8	20/F	T-cell acute lymphoblastic leukemia	15¶	10¶	0	0	24	0	Died (day 24)	Aspergillus infection
9	12/M	Acute myelogenous leukemia	15¶	10¶	10¶	5¶	18	0	Alive, in complete remission (day 321)	
11	0.5/F	Acute myelogenous leukemia	15¶	10¶	10¶	0	23	0	Died (day 60)	Relapse of primary disease
12	16/M	T-cell acute lymphoblastic leukemia	15¶	10¶	10¶	5¶	14	B/day 49	Alive, in complete remission (day 132)	

*HSCT denotes hematopoietic stem-cell transplantation, GVHD graft-versus-host disease, and NE unable to be evaluated.

†Patients were considered to have “good-risk” disease if they had leukemia and were in a second remission, if they had lymphoma and were in remission, or if they had myelodysplasia without excess blasts or marrow failure. All others were considered to have “poor-risk” disease.

‡The time to engraftment is defined here as the time to the first of three consecutive days in which there was an absolute neutrophil count of ≥500 per microliter.

§Severity was determined according to the criteria of the International Bone Marrow Transplant Registry.³⁵

¶Treatment was followed by leucovorin rescue. In Patients 9, 10, 11, and 12, the amended protocol specified a mandatory 50 percent reduction in the dose of methotrexate to 5 mg per square meter of body-surface area on day 11 as well as mandatory rescue with leucovorin after all doses of methotrexate.

||This patient’s transplant engrafted 20 days after the second marrow infusion (on day 55 after the initial transplant was infused).

Ex Vivo Induction of Anergy

Cryopreserved mononuclear cells derived from the recipient’s blood were thawed, washed, and irradiated at a midplane dose of 3300 cGy. The cells were resuspended at a concentration of 20 million cells per milliliter in RPMI 1640 medium with 5 percent human AB serum. CTLA-4-Ig (Repligen, Cambridge, Mass.) was added at a concentration of 20 µg per milliliter for a minimum of 30 minutes before the addition of the donor marrow cells. The erythrocyte-depleted, mononuclear-cell fraction of the marrow was resuspended at a concentration of 20 million cells per milliliter in RPMI 1640 medium with 5 percent human AB serum and added to the mixture of recipient cells and CTLA-4-Ig in a donor: recipient ratio of 1:1 to 2:1. Cells were cultured in Lifecell tissue-culture flasks (Baxter, Deerfield, Ill.) for 36 hours at 37°C in 5 percent carbon dioxide, washed, and then infused into the recipient.

Immunologic Assays

The frequency of precursor helper T cells was determined as previously reported.³² Briefly, we irradiated (with 2500 cGy) 1 million stimulator cells per milliliter in RPMI 1640 medium with 5 percent heat-inactivated human AB serum; 100 µl of the culture medium was then added to each well of a U-bottomed microtiter plate.

Nonirradiated responder cells were added in aliquots of 100 µl to each of 72 replicate wells at dilutions ranging from 100,000 cells to 1 cell per well. After incubation for 64 hours at 37°C in 5 percent carbon dioxide, plates were irradiated (with 2500 cGy), CTLL-2 cells (interleukin-2-responsive T cells; 1000 cells in a volume of 50 µl) were added, and the culture was continued for another 24 hours. Proliferation was assessed by the incorporation of tritiated thymidine for the last 16 hours. The frequency of precursor cells was calculated on the basis of the proportion of wells showing no production of interleukin-2 by limiting-dilution analysis. Stimulation indexes were determined by mixed-leukocyte culture.³² In vitro production of antibodies with specificity against influenza A was determined.³⁷ Influenza-specific antibodies were quantitated by enzyme-linked immunosorbent assay.

Donor-Recipient Hematopoietic Chimerism

Hematopoietic chimerism was evaluated by fluorescence in situ hybridization for sex chromosomes or by polymerase-chain-reaction (PCR) amplification of sequence-specific primers or oligonucleotide probes for HLA class I and class II donor and recipient antigens. Unfractionated blood or marrow samples were analyzed. In some cases, after Ficoll-Hypaque separation and red-cell lysis, the pellet was analyzed as the “granulocyte” fraction and the lym-

TABLE 4. CHARACTERIZATION OF INFUSED DONOR CELLS.

PATIENT No.	NO. OF CELLS REINFUSED/kg OF RECIPIENT'S BODY WEIGHT				
	MONONUCLEAR CELLS ($\times 10^{-8}$)	CD34+ ($\times 10^{-6}$)	CD3+ ($\times 10^{-7}$)	CD4+ ($\times 10^{-7}$)	CD8+ ($\times 10^{-7}$)
1	2.4	6.5	5.5	4.1	1.8
2	1.3	2.0	1.7	1.3	0.7
3	0.9	2.1	1.6	1.1	0.6
4*					
Infusion 1	0.7	2.3	3.2	2.1	1.0
Infusion 2	0.2	0.6	0.9	0.5	0.4
5	1.2	NE†	2.6	1.0	0.9
6	0.7	1.3	1.2	0.4	0.7
7	0.6	1.3	1.2	0.9	0.2
8	2.4	3.7	5.2	2.8	2.0
9	1.3	1.8	4.2	1.6	1.0
10	1.3	3.9	2.0	1.1	0.7
11	1.5	6.0	4.7	2.2	2.2
12	0.7	NE†	3.1	1.6	1.0
Median	1.3	2.2	2.8	1.4	0.9

*Patient 4 received two anergic bone marrow infusions from the same donor, the first on day 0 and the second on day 35.

†NE denotes not able to be evaluated. The products of the peripheral-blood leukaphereses of Patients 5 and 12 were heavily contaminated with CD34+ leukemic cells, rendering the final CD34+ determination uninformative with respect to the stem-cell content of the infused marrow.

phomonuclear interface was analyzed as the "lymphocyte" fraction in order to differentiate between myeloid and lymphoid cells. PCR, as performed here with sequence-specific primers for class I and class II alleles, can detect 1 in 100 and 1 in 10,000 cells, respectively.³⁸ Although expected to be substantially greater, the sensitivity of sequence-specific oligonucleotide probes in this setting has not been defined.

Detection of Leukemic Cells after Transplantation

In patients with a previously documented cytogenetic abnormality, specimens of bone marrow aspirate were sent to the clinical cytogenetic laboratory for analysis. To identify gene rearrangements associated with leukemia and rearrangements of the immunoglobulin gene in patients with acute lymphoblastic leukemia, genomic DNA was extracted from samples taken from the patients and amplified by PCR with a series of seven consensus primers for the framework-1 segment of the V_H (variable heavy chain) region and a J_H (joining heavy chain) consensus primer, as previously described.³⁹ Clonal PCR products were excised and purified with Wizard PCR Preps (Promega, Madison, Wis.). Purified PCR fragments were sequenced directly by the Molecular Biology Core Facility at the Dana-Farber Cancer Institute with a DNA sequencer (model AB 373A, Perkin Elmer Applied Biosystems, Foster City, Calif.). The relevant V_H family and J_H consensus primers were used to obtain the sequence information from both strands, and sense and antisense sequences were aligned. V_H , D_H (diversity heavy chain), J_H , and regions of the N-nucleotide addition were identified with the Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, Md.).

Antisense allele-specific oligonucleotide primers for the CDR-III region were designed to have annealing temperatures of approximately 60°C and were synthesized by GIBCO BRL (Gaithersburg, Md.). Seminested PCR was performed as previously described.⁴⁰ PCR products were then analyzed by electrophoresis on 2 percent agarose gels. Well-established precautions were taken to prevent carryover contamination of PCR reactions.⁴¹

The cellular immunophenotype was determined by dual-color

fluorescence-activated cell sorting (Coulter Profile, Coulter, Miami) with the use of directly conjugated monoclonal antibodies for CD3+, CD4+, CD8+, CD56+, CD20+ (Coulter), and CD34+ (Becton Dickinson, San Jose, Calif.).

Statistical Analysis

Descriptive statistics are provided for determinations of the frequency of precursor cells. The Wilcoxon signed-rank test was used to assess changes in the logarithm (base 10) of the assay results before and after treatment with CTLA-4-Ig.

RESULTS

Frequency of Alloreactive Precursor Helper T Cells after Induction of Anergy

The harvested donor bone marrow contained mature T cells, with medians of 16 million CD3+ cells per kilogram of the recipient's weight, including 8 million CD3+CD4+ cells per kilogram and 6 million CD3+CD8+ cells per kilogram. Medians of 28 million CD3+ cells per kilogram, including 14 million CD3+CD4+ cells per kilogram and 9 million CD3+CD8+ cells per kilogram, were infused after the ex vivo treatment (Table 4). Because not all of the irradiated recipient T cells were removed by density centrifugation after the coculture procedure, the number of T cells infused into the patient was generally greater than that in the harvested marrow.

The reactivity of donor T cells against alloantigens from the designated recipient and persons unrelated to the recipient or the donor (third parties) was assessed by determining the frequency of helper T-cell precursors before and after culture with soluble

CTLA-4-Ig and irradiated mononuclear cells from the recipient. As Figure 1 shows, the frequency of precursor helper T cells from unmanipulated donor bone marrow with alloreactivity against haploidentical recipient cells ranged from 0.0002 to 0.002 (median, 0.0005). Because donors and third parties are unlikely to share haplotypes of major histocompatibility antigens, the frequency of precursor helper T cells with alloreactivity against cells from third parties was higher, although not significantly so (range, 0.0006 to 0.004; median, 0.001). After the in vitro treatment to induce anergy, the frequency of precursor helper T cells that were alloreactive against haploidentical cells from the recipient fell by one to four orders of magnitude (range, 0.0000001 to 0.00002; median, 0.0000009; $P=0.008$), whereas the frequency of precursors with alloreactivity against cells from third parties was not significantly affected (range, 0.00009 to 0.001; median, 0.0008; $P=0.51$).

Myeloid Engraftment and Chimerism after Transplantation of Anergic Haploidentical Bone Marrow

A median of 3 million CD34+ donor cells per kilogram of the recipient's weight were treated in the coculture system, and a median of 2.2 million CD34+ cells per kilogram were infused into the recipient (Table 4). A second marrow harvest from the donor of Patient 4 yielded 900,000 CD34+ cells per kilogram of the recipient's weight, and 600,000 CD34+ cells per kilogram were infused.

Patient 2, who died on day 8, could not be evaluated for engraftment (Table 3). In Patient 4 the neutrophil count did not reach 500 per microliter despite cytogenetic evidence of donor cells in the recipient's bone marrow and blood. Therefore, cells from a second harvest were treated ex vivo and infused on day 35 without further conditioning of the patient or prophylaxis against GVHD. The neutrophil count was more than 500 per microliter 20 days later. Patient 1 achieved a neutrophil count of 100 per microliter on day 21, one day before her death. The remaining patients had prompt recovery of neutrophils (a median of 20 days until a neutrophil count ≥ 500 per microliter was reached, Table 3). Patients 1, 2, 4, 5, 6, and 8 died before platelet or red-cell engraftment could be documented. Patients 3, 7, 10, 11, and 12 achieved a platelet count of more than 20,000 per microliter between 22 and 123 days after transplantation (median, 43). Patient 11 died before confirmed red-cell engraftment.

Patients 3, 7, 10, and 12 received their last red-cell transfusion 51 to 101 days after transplantation (median, 65). Patient 9 had persistent moderate pancytopenia, presumed to be secondary to recurrent cytomegalovirus infection. He received his last red-cell transfusion on day 108 and required intermittent platelet transfusions until day 197. The other surviving patients all have normal blood counts. Chimer-

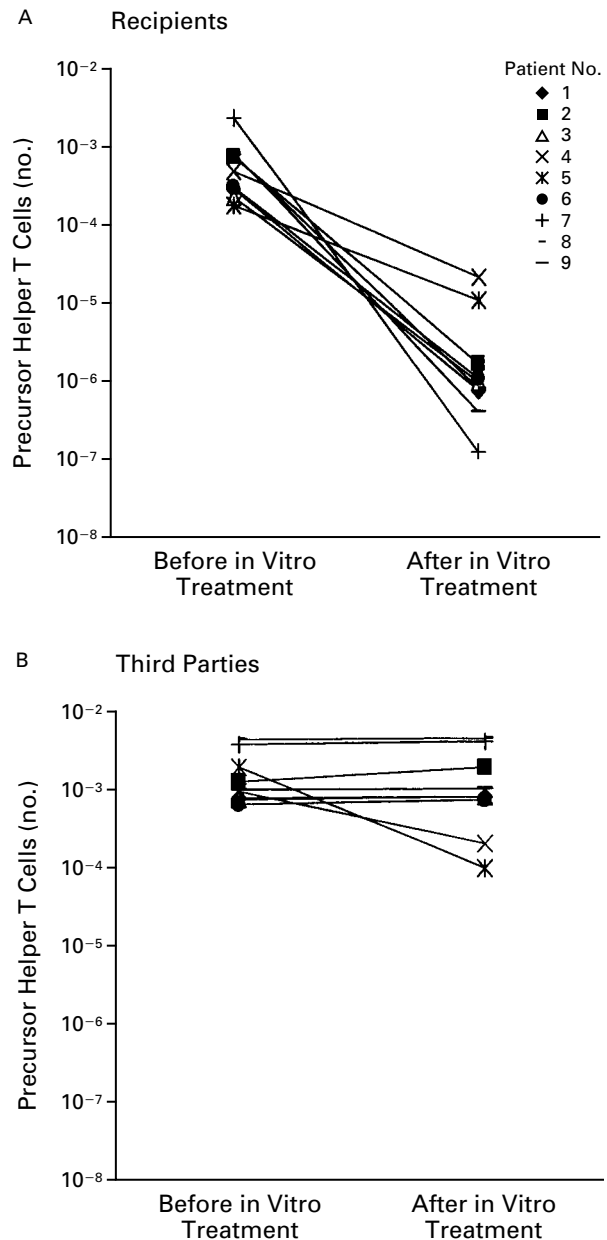


Figure 1. Frequency of Precursor Helper T Cells before and after Induction of Anergy.

The frequency of precursor helper T cells with alloreactivity against irradiated cells from the recipient (Panel A) and against cells from persons unrelated to the recipient or the donor (third parties; Panel B) was calculated before and after the induction of anergy in the cells of donors. Insufficient numbers of cells were available to perform this analysis for Patients 10, 11, and 12.

ism studies, performed in 8 of 10 patients in whom engraftment occurred, revealed no evidence of residual recipient hematopoietic cells at the first or subsequent evaluations (Table 5).

GVHD

Despite the possibility of ex vivo sensitization of donor T cells against recipient alloantigens, no hyperacute GVHD was observed.⁴² Eleven of the 12 patients could be evaluated for acute GVHD, and 6 patients could be evaluated through day 100 (the entire risk period for acute GVHD). None of the patients had skin or liver findings consistent with acute GVHD (Table 3), but three (Patients 3, 5, and 11) had gastrointestinal symptoms and findings consistent with acute GVHD. Two of these patients had only diarrhea, and one had gastrointestinal hemorrhage. These nonspecific events might have been caused by concurrent influenza A (in Patient 3), persistent gastrointestinal toxic effects at day 20 from chemoradiotherapy (in Patient 5), and documented intolerance of infant formula (in Patient 11).

Nonetheless, on the basis of stool volume and biopsy specimens showing apoptosis or degeneration of crypt cells, Patient 3 was considered to have GVHD with an overall IBMTR index rating of B (skin and liver involvement, stage 0; gastrointestinal involvement, stage 2). This condition resolved within 10 days after treatment with methylprednisolone was begun. Patient 11, who had GVHD with an overall rating of B (skin and liver involvement, stage 0; gastrointestinal involvement, stage 2), also had a response to corticosteroids. Patient 5 had GVHD with an overall rating of C (skin and liver involvement, stage 0; gastrointestinal involvement, stage 3). Her stool output diminished temporarily with corticosteroid treatment, then diarrhea persisted until death on day 39. An autopsy disclosed ischemia and hemorrhage, without evidence of GVHD, suggesting that the GVHD had been successfully treated or that the patient had not had GVHD. Limited autopsy (Patient 4) or complete autopsy (Patients 2, 5, and 6) did not reveal pathological evidence of GVHD.

Only one of the patients given cytarabine for conditioning received all of the intended GVHD prophylaxis. Only half of the patients given cytarabine received the intended dose of methotrexate on day 3, only two of eight did so on day 6, and none of the seven patients who could be evaluated did so on day 11 (Table 3). It was anticipated that cyclosporine would be continued for 6 to 12 months after transplantation. As the study progressed, the target trough level of cyclosporine was decreased from 200 to 300 mg per milliliter to 100 to 150 mg per milliliter, and cyclosporine was discontinued much earlier than expected (on days 289, 88, 122, 101, and 120 for Patients 3, 7, 9, 10, and 12, respectively).

TABLE 5. FULL HEMATOPOIETIC CHIMERISM IN RECIPIENTS AFTER TRANSPLANTATION.*

PATIENT NO.	TYPE OF SAMPLE	DAY AFTER TRANSPLANTATION	METHOD†
1	Peripheral blood	21	FISH
	Granulocytes	21	FISH
3	Bone marrow aspirate	134	SSOP, SSP
	Peripheral-blood lymphocytes	634	SSP
	Granulocytes	634	SSP
	Peripheral-blood lymphocytes	754	SSP
7	Peripheral blood	64	SSOP
	Peripheral blood	173	SSOP, SSP
	Peripheral-blood lymphocytes	329	SSP
	Granulocytes	329	SSP
8	Peripheral blood	20	Cytogenetics
	Peripheral blood	29	SSOP, SSP
9	Bone marrow aspirate	79	SSOP
	Peripheral-blood lymphocytes	122	SSP
	Granulocytes	122	SSP
	Peripheral-blood lymphocytes	198	SSOP
10	Peripheral blood	24	SSP
	Peripheral-blood lymphocytes	122	SSP
	Granulocytes	122	SSP
11	Peripheral blood	16	SSOP, SSP
	Peripheral blood	24	SSP
12	Peripheral blood	31	SSP

*All samples from these patients were demonstrated to have 100 percent donor cells by the methods listed at each evaluation.

†FISH denotes fluorescence in situ hybridization, SSOP PCR with sequence-specific oligonucleotide probes, and SSP PCR with sequence-specific primers.

Clinical Outcome

Outcome was assessed according to disease classification (Table 3). Of the good-risk patients, one died early of regimen-related toxicity and three are alive, disease-free, and at home receiving no cyclosporine, corticosteroids, or other immunosuppressive treatment; they have no signs of chronic GVHD. Patient 10, who received a bone marrow transplant for congenital amegakaryocytopenia, has normal hematopoiesis and a platelet count of more than 250,000 per microliter. Neither Patient 3 nor Patient 7 has clinical evidence of leukemic relapse, and no evidence of leukemia was detected by PCR analysis of bone marrow samples (from day 754 for Patient 3 and day 391 for Patient 7) with the use of allele-specific oligonucleotide probes for rearrangements present in the patients' pretransplantation leukemic cells.

One of the eight poor-risk patients died on day 60 of a relapse of acute myelogenous leukemia. Five

patients, including Patients 2 and 6, who had previously undergone autologous transplantation, died of infection and regimen-related toxicity before or shortly after day 100. Autopsies of Patients 2, 4, 5, and 6 disclosed no histologic evidence of cancer. Two poor-risk patients are alive and at home. In Patient 9, diarrhea and recurrent cytomegalovirus infection developed after discharge. Biopsy specimens taken from the small bowel and sigmoid colon on day 145 revealed GVHD, classified as chronic by virtue of its occurrence after day 100, but a biopsy of a rash showed no evidence of GVHD. Patient 9 was treated with cyclosporine and corticosteroids, with complete histologic resolution shown by a repeated biopsy of the gastrointestinal tract on day 197. The doses of corticosteroids and cyclosporine are being tapered. A bone marrow aspirate obtained from Patient 9 on day 197 was histologically normal and free of the prior cytogenetic abnormality (inversion of chromosome 12). As of this writing, Patient 12 is at day 132, two weeks after discontinuing cyclosporine, and has had no evidence of acute or chronic GVHD. Analysis of blood obtained from this patient on day 67 showed no evidence of leukemia on PCR amplification of allele-specific oligonucleotide primers for the T-cell-receptor rearrangement present in his pretransplant leukemic cells.

Infection and Immune Reconstitution

Infections developed in three patients after discharge. Influenza A developed in Patient 3 on day 56, after he was exposed to his infected sibling. It resolved without incident after treatment with amantadine. Dermatomal herpes zoster also developed on day 509 and was treated uneventfully with acyclovir. Patient 9 received his transplant immediately after an episode of cytomegalovirus viremia and acute hepatic necrosis. Recurrent cytomegalovirus infection developed on day 36 and was treated successfully with immune globulin and foscarnet. Another recurrence of cytomegalovirus infection soon after discharge was also treated successfully. Patient 10, who is currently in day care, was given a diagnosis of community-acquired respiratory syncytial virus on the basis of screening of nasal secretions nine months after transplantation. The nasal congestion resolved uneventfully without treatment.

As assessed by the expression of CD56, all the survivors except Patient 9 (Patients 3, 7, 10, and 12) had increased numbers of natural killer cells soon after transplantation. They also rapidly recovered and maintained normal numbers of CD20+ B cells. IgG levels were maintained in the age-adjusted normal range by 9 months in Patient 3, 3.5 months in Patient 7, 2.5 months in Patient 10, and 3 months in Patient 12. Patient 9 could not be evaluated because he received immune globulin for prophylaxis against cytomegalovirus infection.

By two months after transplantation, the CD3+CD4+:CD3+CD8+ ratio was ≥ 1 in all the patients evaluated (Patients 7, 9, 10, and 12). Patient 3, first evaluated four months after transplantation, also had a ratio of 1. All five surviving patients had a normal CD3+CD4+:CD3+CD8+ ratio (≥ 1.4) by seven months after transplantation. The absolute CD4+ T-cell counts rose to 400 per microliter or higher by six months in three of the four patients who survived until then; the sole exception was Patient 9, who had recurrent cytomegalovirus disease and chronic GVHD. Currently, Patient 12 has an absolute CD4+ T-cell count of 140 per microliter four months after transplantation.

Engrafted donor T cells from Patients 3 and 7 have been assessed for reactivity against recipient alloantigens. Peripheral-blood mononuclear cells collected from Patient 3 on day 379, 90 days after cyclosporine was discontinued, were cultured with either irradiated stimulator cells from three unrelated persons or with irradiated cryopreserved recipient peripheral-blood cells. The proliferative response to the cells from the unrelated persons was 5 to 11 times as high on day 5 of coculture as the response to the recipient cells. In an identical experiment with peripheral-blood mononuclear cells collected from Patient 7 on day 173, 85 days after the discontinuation of cyclosporine, the proliferative response to the cells from the unrelated persons was 6 to 12 times as high as the response to the recipient cells. In concurrent experiments, the frequencies of helper T-cell precursors responding to cryopreserved cells were 0.00001 in Patient 3 and 0.0000008 in Patient 7, as compared with frequencies of precursors responding to cells from unrelated persons of 0.0003 and 0.00003, respectively.

Community-acquired influenza A in Patient 3 provided an opportunity to measure the *in vivo* response to an infection that had not affected the recipient or donor before transplantation. Immediately before vaccination of both the recipient and the donor against influenza A on day 355, the recipient's engrafted cells responded to the influenza A virus *in vitro*, presumably because he had a naturally acquired infection two months after transplantation, whereas the cells from the uninfected donor showed no response. After vaccination, both the recipient and the donor had measurable responses *in vitro*.

DISCUSSION

We found that *ex vivo* blockade of B7-mediated costimulation induces anergy in T cells residing in the bone marrow of a haploidentical donor that are ordinarily reactive to alloantigens of the recipient. Moreover, this treated marrow, after transplantation, supports the hematologic reconstitution of the recipient and replaces all of the recipient's hematopoietic cells. With conventional prophylaxis against GVHD,

we would have expected to see severe GVHD in 60 to 90 percent of recipients of haploidentical marrow,^{43,44} yet only one case of acute GVHD with an IBMTR rating of C and no cases with a rating of D were observed, and no deaths were attributable to GVHD. Prophylaxis against GVHD could be discontinued uneventfully, and chronic GVHD has developed in only one patient. Despite an anticipated high rate of mortality in this population, 5 of the 12 patients are alive and disease-free.

It appears that donor marrow treated *ex vivo* to induce anergy can reconstitute hematopoiesis with full lymphoid and myeloid donor chimerism (Table 5). This study did not have adequate power for statistical certainty but, rather, was designed to investigate whether the expected high incidence of GVHD would be diminished after the *ex vivo* treatment. The occurrence of only two cases of acute GVHD with a rating of B and one case with a rating of C was particularly striking, since all the patients had multiple risk factors for GVHD, of which donor–recipient histoincompatibility was the most obvious.^{43,45–47} Furthermore, all the patients received donor bone marrow containing more than 10 million CD3+ T cells per kilogram of the recipient's weight, exceeding by one to two orders of magnitude the suggested threshold dose for minimizing GVHD.^{48,49} As a group, the 12 patients we treated had additional risk factors for GVHD, including mismatching of donor and recipient with respect to sex, donors who had been pregnant, incomplete methotrexate or cyclosporine prophylaxis, recurrent or persistent disease, irradiation-based conditioning (particularly with >1200 cGy), cytomegalovirus seropositivity, and older donor age.^{43,46,50–55} Moreover, the onset of GVHD is often early in cases of transplantation of haploidentical donor marrow with conventional immunoprophylaxis.^{56,57} All these elements support the contention that *ex vivo* induction of anergy in donor marrow T cells can prevent or minimize GVHD.

An alternative approach to the problems of graft failure and severe GVHD after haploidentical marrow transplantation involves intensive myeloablation with multiple agents, coupled with treatment of the recipient with antithymocyte globulin before transplantation, exhaustive depletion of T cells from the donor's marrow, and markedly increased doses of donor stem cells. Partial depletion of T cells and immunosuppression after transplantation, including that resulting from treatment with antithymocyte globulin, have also been tried.^{58–61} However, graft failure, substantial regimen-related toxicity, opportunistic infection, and delayed reconstitution of immune function are still problems.^{58–62} Because these approaches rely on nonspecific ablation of T cells, immunosuppression, and aggressive preparation of the recipient, these obstacles will probably be difficult to overcome.

As indicated by the frequency of precursor helper T cells with alloreactivity against haploidentical re-

ipient alloantigens in the recipient–donor pairs that we studied, the frequency of donor helper T-cell precursors responsive to haploidentical recipient alloantigens is at most 0.1 percent (i.e., 0.001). This frequency suggests that more than 99 percent of the 500 million to 1.5 billion cells contained in the harvested donor marrow do not respond to recipient alloantigens and may be infused without the recipient's incurring a substantial risk of GVHD. This strategy of inducing anergy affects only a particular segment of the T-cell repertoire, leaving the rest to aid engraftment and restore immunocompetence. On the basis of recent assessments in this small cohort of patients, it appears that there is potential for rapid recovery of a normal ratio of CD4+ T cells to CD8+ T cells in peripheral blood and that the absolute number of CD4+ T cells can reach a normal level by approximately six months after transplantation.

More important, patients appear able to overcome acquired and reactivated viral infections. No lymphoproliferative disease associated with the Epstein–Barr virus has been seen clinically or on autopsy thus far, despite nearly uniform seropositivity in the patients and the donors. There were, however, four deaths related to infection. One patient died of bacterial sepsis (Patient 2), and two died of aspergillus infection (Patients 5 and 8). These infections occurred very soon after transplantation and only in patients with chronic neutropenia due to relapsed disease and severe mucositis who had received cytarabine as part of their conditioning regimen. Patient 4, another member of the group that received cytarabine, died of disseminated toxoplasmosis, discovered at autopsy. This patient had protracted leukopenia and neutropenia because of delayed engraftment. There were two deaths resulting from multiorgan failure in this cohort. No fungal disease, deaths from infection, or episodes of multiorgan failure have occurred since cytarabine has been eliminated from the conditioning regimen, suggesting that these events may have been due to the additional immunosuppression, mucositis, and toxic effects resulting from the aggressive conditioning regimen.^{60,61,63}

In summary, we showed that inactivation of a selected segment of the alloreactive T-cell repertoire through blockade of B7-mediated T-cell costimulation is possible and appears to result in a significant reduction in the risk of GVHD. Further evidence that the portion of the T-cell repertoire that is not alloreactive is left unperturbed is essential in establishing whether this and similar methods are realistic alternatives to solving the difficult problem of transplanting haploidentical bone marrow.

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