

Nonmyeloablative Bone Marrow Transplantation from Partially HLA-Mismatched Related Donors Using Posttransplantation Cyclophosphamide

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ABSTRACT

Cyclophosphamide (Cy) is a potent immunosuppressive agent that is selectively toxic to lymphocytes proliferating in response to recent antigen stimulation. In animal models, both graft rejection and GVHD after histoincompatible BMT can be inhibited by the posttransplantation administration of high-dose Cy. Therefore, a phase I clinical trial was undertaken to determine the minimal conditioning, including posttransplantation Cy, that permits the stable engraftment of partially HLA-mismatched marrow (up to 3 HLA antigens) from first-degree relatives. Thirteen patients (median age, 53 years) with high-risk hematologic malignancies received conditioning with fludarabine, 30 mg/m² per day from days -6 to -2, and TBI, 2 Gy on day -1. All patients received Cy, 50 mg/kg on day 3, mycophenolate mofetil from day 4 to day 35, and tacrolimus from day 4 to day ≥50. Three patients in cohort 1 received no additional conditioning, and 2 experienced graft rejection. Ten patients in cohort 2 received identical conditioning with the addition of Cy 14.5 mg/kg on days -6 and -5. Sustained donor cell engraftment occurred in 8 of these patients, with a median time to absolute neutrophil count >500/μL of 15 days (range, 13-16 days) and to unsupported platelet count >20,000/μL of 14 days (range, 0-26 days). All patients with engraftment achieved ≥95% donor chimerism within 60 days of transplantation. Two patients with myelodysplastic syndrome rejected their grafts but experienced autologous neutrophil recovery at 24 and 44 days. Histologic acute GVHD developed in 6 patients (grade II in 3 patients, grade III in 3 patients) at a median of 99 days (range, 38-143 days) after transplantation and was fatal in 1 patient. At a median follow-up of 191 days (range, 124-423 days), 6 of 10 patients in cohort 2 were alive, and 5 were in complete remission of their disease, including both patients with graft rejection. These data demonstrate that partially HLA-mismatched bone marrow can engraft rapidly and stably after nonmyeloablative conditioning that includes posttransplantation Cy. Clinically significant antitumor responses occur, even among patients who reject their donor grafts.

KEY WORDS

Nonmyeloablative bone marrow transplantation • Cyclophosphamide • HLA-mismatched related donors

INTRODUCTION

The combination of high-dose chemoradiotherapy and allogeneic blood or bone marrow transplantation (BMT) can cure a variety of hematologic malignancies but is not an option for many patients who lack an HLA-matched donor, either related or unrelated. Although there is a greater than 95% chance that any patient has a living partially HLA-

mismatched, or "haploidentical," first-degree relative [1], 2 or 3 HLA antigen-mismatched BMT has been complicated by high rates of fatal graft rejection, significant graft-versus-host disease (GVHD), and transplantation-related mortality [2-8]. Thus, development of a tolerable approach to HLA-haploidentical BMT that limits graft rejection and GVHD is an important goal, because the graft-versus-leukemia effect may be augmented in this setting [2,4].

A major goal of nonmyeloablative stem cell transplantation is to minimize conditioning-regimen toxicity. Moreover, the use of nonmyeloablative conditioning for HLA-mismatched

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BMT would carry the additional safeguard of recovery of autologous hematopoiesis in the event of graft rejection. For example, Storb and colleagues have found that posttransplantation administration of cyclosporine A (CsA) and mycophenolate mofetil (MMF) permits engraftment of major histocompatibility complex (MHC)-identical allogeneic bone marrow in dogs conditioned with only 200 cGy total body irradiation (TBI) [9]. When this strategy was applied to patients receiving HLA-identical sibling peripheral blood grafts, a 20% incidence of nonfatal graft rejection was observed [10]. However, the incidence of graft rejection in this setting was reduced to 3% when a 3-day course of pretransplantation fludarabine was added to low-dose TBI and posttransplantation CsA and MMF [11]. However, nonmyeloablative conditioning is unlikely to be sufficient for preventing rejection of HLA-mismatched allogeneic transplants. Ildstad and colleagues found that posttransplantation high-dose cyclophosphamide (Cy) permitted engraftment of MHC-incompatible marrow grafts after nonmyeloablative conditioning in mice [12,13]. Owens and Santos had found earlier that posttransplantation high-dose Cy also effectively prevented GVHD in mice [14]. Because of its potent immunosuppressive properties, Cy has been the backbone of most pretransplantation high-dose conditioning regimens for allogeneic BMT [15-17], but concerns about its myelotoxicity have deterred clinical application of high-dose Cy in the posttransplantation setting. However, it is now clear that high-dose Cy does not induce myeloablation; full autologous hematopoietic recovery occurs rapidly when transplantation doses of Cy are used alone as treatment for autoimmune disorders [18]. The unique pharmacology of high-dose Cy is responsible for its ability to induce maximal immunosuppression without myeloablation. Hematopoietic stem cells express high levels of aldehyde dehydrogenase, an enzyme responsible for cellular resistance to Cy, whereas B-lymphocytes, T-lymphocytes, and natural killer (NK) cells express low levels of the enzyme and are extremely sensitive to the cytotoxic properties of Cy [19]. The use of high-dose Cy early after BMT, during activation of alloreactive effector cells, may accentuate its cytotoxic activity against both host-versus-graft (HVG) and GVH reactions [20,21].

With this background, we have developed a nonmyeloablative conditioning regimen that uses pretransplantation fludarabine and 200 cGy TBI, with high-dose Cy administered on day 3 after BMT [22]. This regimen permitted stable engraftment of major +/- minor histocompatibility antigen-mismatched marrow, including 3 MHC antigen-mismatched haploidentical marrow, in the vast majority of donor-recipient pairs of mice. In addition to preventing graft rejection, high-dose posttransplantation Cy ameliorated GVHD after MHC-mismatched BMT in mice receiving either myeloablative or nonmyeloablative conditioning. Addition of pretransplantation Cy to the conditioning regimen was also found to increase immunosuppression and augmented donor chimerism, as recently described by Petrus et al. [23]. Based on these data, we initiated a clinical trial in patients with high-risk hematologic malignancies undergoing nonmyeloablative BMT from HLA-haploidentical donors to determine the effective dose of pretransplantation Cy required for full donor engraftment. To reduce the risk of GVHD, all patients received

posttransplantation immunosuppression with high-dose Cy on day 3 plus the potent combination of MMF and tacrolimus [10,24]. Because calcineurin inhibitors, such as CsA or tacrolimus, inhibit Cy-induced immunologic tolerance [25], treatment with tacrolimus and MMF was delayed until day 4 after BMT.

PATIENTS AND METHODS

Patients

Eligible patients were 0.5 to 70 years of age with high-risk hematologic malignancies for whom standard allogeneic BMT (HLA-matched, related or unrelated) or autologous BMT were unavailable or inappropriate. Eligible diagnoses included interferon-refractory chronic myeloid leukemia (CML) in first chronic phase, CML in second chronic phase, poor-risk acute leukemia in first complete remission, acute leukemia in second or subsequent remission, myelodysplastic syndrome (MDS), and chemotherapy-resistant lymphoma or multiple myeloma. Patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. Criteria for organ function were forced expiratory volume (FEV₁) \geq 40% predicted, left ventricular ejection fraction \geq 35%, and total bilirubin \leq 3.0 mg/dL. Informed consent was obtained from patients using forms approved by the Joint Committee on Clinical Investigation of the Johns Hopkins University.

HLA Typing

HLA class I typing was performed by microcytotoxicity using a combination of locally procured and commercially obtained typing sera for patients and donors enrolled prior to July 1, 2000. Subsequently, all class I typing was performed by sequence-specific primer amplification (SSP) using commercial primers (Pel-Freez Clinical System, Brown Deer, WI) to define intermediate-resolution HLA-A, B, and Cw allele groups. In 3 cases, sufficient family members were available for genotyping and only low-resolution typing for DRB1 was done by SSP (One Lambda, Canoga Park, CA). In all other cases, DRB1 alleles were defined by reverse sequence-specific oligonucleotide probe (SSOP) hybridization (LiPA, Innogenetics, Gent, Belgium) or by automated cycle sequencing using dye terminator chemistry analyzed on an ABI 377PRISM DNA sequencer (Applied Biosystems, Foster City, CA). DQB1 alleles were typed to intermediate resolution by reverse SSOP. All molecular typing was done from genomic DNA samples extracted from peripheral blood lymphocytes (QIAamp blood kit, Qiagen, Chatsworth, CA).

Trial Design

The major objective of the clinical trial was to determine the minimum dose of pretransplantation Cy for prevention of allograft rejection. The dose of Cy was titrated according to a modified method of continual reassessment [26]. The first cohort of 3 patients received no pretransplantation Cy. In the absence of efficacy, defined as graft rejection in 2 of the first 2 or 3 patients, the dose of pretransplantation Cy was escalated. Ten patients in the second treatment cohort received Cy 14.5 mg/kg per day on days -6 and -5.

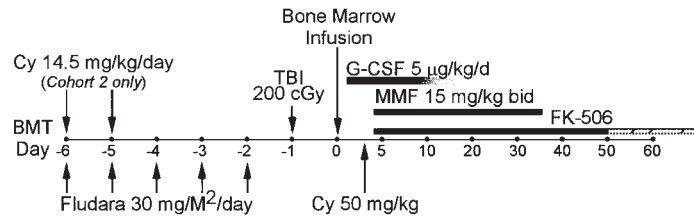


Figure 1. Treatment schema. All patients received fludarabine from days -6 to -2 , 200 cGy TBI on day -1 , a haploidentical bone marrow infusion on day 0, Cy on day 3, and MMF on days 4 to 35. Granulocyte colony-stimulating factor (G-CSF) treatment was discontinued when the ANC exceeded $1000/\mu\text{L}$ for 3 consecutive days. All patients in cohort 2 received Cy on days -6 and -5 ; the last 2 patients of this cohort received tacrolimus beyond day 100.

Transplantation Regimen

Patients received conditioning therapy with fludarabine $30\text{ mg}/\text{m}^2$ per day intravenously (IV) on days -6 to -2 [27] followed by 200 cGy of TBI on day -1 [9,12,13,28] (Figure 1). The patients then received a marrow allograft (depleted of red blood cells and plasma by processing on a Gambro Spectra apheresis instrument) on day 0. On day 3, 50 mg/kg Cy was administered over 90 minutes together with Mesna (80% dose of Cy in 4 divided doses over 8 hours) by IV infusion. The first 9 patients (3 patients in cohort 1 and 6 patients in cohort 2) received MMF (Cellcept; Roche Laboratories, Nutley, NJ) 15 mg/kg orally twice a day from days 4 to 35 and tacrolimus (Prograf; Fujisawa, Deerfield, IL) from days 4 to 50. Tacrolimus was initiated at a dose of 1 mg IV daily, adjusted to achieve a therapeutic level of 5 to 15 ng/mL, and then converted to oral form until discontinuation. Because GVHD developed after day 100 in 3 of the first 6 patients of cohort 2, a decision was made to extend tacrolimus treatment in all patients with $\geq 95\%$ donor chimerism at day 60. Patients received filgrastim (Neupogen; Amgen, Thousand Oaks, CA), 5 $\mu\text{g}/\text{kg}$ per day by subcutaneous injection starting on day 1 and continuing until the recovery of neutrophils to $>1000/\mu\text{L}$ for 3 days. Prophylactic antimicrobial therapy was started on day -6 and included norfloxacin 400 mg orally twice daily, fluconazole 400 mg orally daily, appropriate prophylaxis for *Pneumocystis carinii* pneumonia, and valacyclovir 500 mg orally thrice daily, as described previously [29]. Cytomegalovirus (CMV) antigenemia was monitored weekly if recipients and/or donors were seropositive. If patients were found to be antigenemic, preemptive treatment with ganciclovir, 5 mg/kg IV twice daily, was initiated for 14 days and then continued daily until CMV antigenemia test results were negative for at least 2 weeks. All treatment was performed on an outpatient basis unless otherwise specified.

Engraftment and Donor Chimerism

Neutrophil recovery was defined as an absolute neutrophil count (ANC) greater than $500/\mu\text{L}$, and platelet recovery was defined as a platelet count greater than $20,000/\mu\text{L}$ for 7 days without transfusion. Donor chimerism was determined either by restriction fragment length polymorphisms [30], polymerase chain reaction analysis of variable nucleotide tandem repeats [31,32], or fluorescence in situ hybridization (FISH) [33] using X- and Y-chromosome probes, if informative. In patient 3267, XY-FISH was performed on peripheral blood mononuclear cells after cellular subsets were fractionated on a MACS separation device

(Miltenyi Biotech, Auburn, CA) using appropriate monoclonal antibodies conjugated to immunomagnetic beads. Date of last patient follow-up was April 9, 2002.

Immune Reconstitution

Peripheral blood lymphocyte subsets were quantified by flow cytometry using monoclonal antibodies to T-cells (CD3, -4 , -8) or NK cells (CD56). Measurements were made at baseline, prior to administration of the conditioning regimen, and weekly after neutrophil counts had recovered to $>1000/\mu\text{L}$ after BMT.

Therapy of GVHD

Acute GVHD was graded according to the Keystone criteria [34]. Treatment approaches are described in the text. Agents used to treat GVHD in addition to methylprednisolone included infliximab (Remicade; Centocor, Malvern, PA), daclizumab (Zenapax; Hoffman-La Roche, Nutley, NJ), and 2-deoxycoformycin (pentostatin, Nipent; SuperGen, San Ramon, CA).

RESULTS

Patient and Donor Characteristics

Table 1 describes the 3 patients in cohort 1 (no Cy before BMT) and the 10 patients in cohort 2 (Cy 14.5 mg/kg each on days -6 and -5). All patients had poor-risk hematologic malignancies and no option of autologous or HLA-identical allogeneic BMT. Median age of recipients was 53 years (range, 23–63 years). HLA typing of the patients and their donors is shown in Table 2. Donors (median age, 37 years; range, 22–63 years) were siblings in 5 cases, children in 6 cases, and parents in 2 cases. Seven donor-recipient pairs were compatible for the ABO blood group antigens. In 2 pairs, recipients had antibodies against donor ABO antigens (a major ABO incompatibility), whereas in 4 pairs, donors had antibodies against recipient ABO antigens (a minor ABO incompatibility). No hemolysis was observed in any of the ABO-incompatible transplantations. All patients had received prior therapy except for 3 patients with MDS: patient 3303 in cohort 1 and patients 3486 and 3529 in cohort 2.

Graft Composition

Allografts consisted of $1.2 \pm 0.1 \times 10^8$ mononuclear cells/kg (mean \pm 95% confidence interval) containing $5.3 \pm 1.5 \times 10^6$ CD34⁺ cells/kg and $3.2 \pm 0.6 \times 10^7$ CD3⁺ cells/kg.

Table 1. Patient Characteristics*

Cohort	UPIN	Age	Sex	Diagnosis
1	3267	56	F	Secondary AML (MDS) in CR1 following induction chemotherapy
	3298	40	M	Ph ⁺ ALL in CR1 following induction and consolidation chemotherapy
	3303	63	F	Hypoplastic MDS; no prior chemotherapy; transfusion-dependent for 10 mo
2	1706	49	M	Stage IV diffuse mixed small cleaved and large cell lymphoma in CR following chemotherapy; relapse after 1 y; CR following chemotherapy, autologous BMT; recurrent abdominal mass, bone lesion after 5 y; CR following radiochemotherapy; recurrence with pathologic fracture of left femoral neck after 3 y; PR following chemotherapy, anti-CD20 therapy
	3384	41	M	CML-CP on interferon therapy; progression to blast crisis after 4 y; CP2 following induction chemotherapy
	3391	53	F	Ph ⁺ ALL in CR1 following induction and consolidation chemotherapy
	3434	60	M	Primary refractory Stage II multiple myeloma following chemotherapy and high-dose dexamethasone
	3271	23	F	M5-AML in CR1 following induction chemotherapy; consolidation by purged autologous BMT; relapse after 6 mo, CR2 following anti-CD33 therapy
	3446	57	F	M1-AML in CR1 following induction and consolidation chemotherapy; relapse after 3 mo; CR2 following anti-CD33 therapy
	3486	38	M	RAEB × 2 y with slow progression to AML; transfusion-dependent × 2 y; no prior therapy
	3529	61	M	Hypoplastic MDS/AA progressing to AML at 19 mo; no prior therapy; red cell and platelet transfusion-dependent
	3545	48	F	CML × 20 mo; interferon × 15 mo without cytogenetic response; MDS, no prior therapy
	3563	53	F	Secondary AML (MDS) in early relapse (by flow cytometry) after induction chemotherapy

*CR indicates complete remission; PR, partial remission; CP, chronic phase; RAEB, refractory anemia with excess blasts; AA, aplastic anemia.

Table 2. HLA Typing

UPIN	Patient Age/Sex	Donor Age (Relationship)	Mismatched HLA Haplotype*					Degree and Direction of HLA Mismatch†			
			Class I Antigen‡			Class II Alleles§		3-Locus Mismatch A;B;DRB1		5-Locus Mismatch A;B;C;DRB1;DQB1	
			A	B	C	DRB1	DQB1	HVG	GVH	HVG	GVH
3267	56/F	25 (Son)	11	51	4	04011	0301	2 (A,B)	3	4 (A,B,C, DQ)	5
			26	49	7	[01xx]	0504				
3298	40/M	34 (Brother)	26	27	2	15011	06WG	3	3	4 (A,B,DR,DQ)	5
			29	60	[7]	1302	06DE				
3303	63/F	38 (Daughter)	24	[7]	[7]	[15011]	[06WG]	3	1 (A)	4 (A,B,DR,DQ)	1 (A)
			32	71	[7]	04011	03TF				
1706	49/M	25 (Son)	11	35	4	03011	02AB	1 (B)	1 (B)	1 (B)	2 (B,C)
			11	8	[7]	03011	02AB				
3384	41/M	37 (Sister)	1	7	7	15011	06xx	1 (A)	1 (A)	1 (A)	1 (A)
			32	7	7	15011	06xx				
3391	53/F	21 (Daughter)	33	50	6	11012	0301	3	3	4 (A,B,DR,DQ)	5
			29	58	[7]	0701	02AB				
3434	60/M	22 (Daughter)	1	58	3	08041	03HGB	2 (A,DR)	2 (A,DR)	3 (A,C,DR)	3 (A,C,DR)
			3	58	6	1102	03HGB				
3271	23/F	55 (Father)	2	35	4	[04011]	03GBP	3	2 (A,B)	5	4 (A,B,C,DQ)
			29	44	16	07xx	02MN				
3446	57/F	55 (Brother)	1	8	7	13011	[06xx]	1 (DR)	1 (DR)	2 (DR,DQ)	1 (DR)
			1	8	7	03011	02MN				
3486	38/M	55 (Mother)	2	70	3	09012	02AB	2 (A,B)	3	4 (A,B,C,DQ)	5
			11	15	4	[0701]	02MN				
3529	61/M	63 (Brother)	2	39	7	0801	04AB	3	3	5	5
			68	53	4	13021	06xx				
3545	48/F	45 (Sister)	1	8	7	03011	02MN	2 (B,DR)	3	4 (B,C, DR,DQ)	5
			[2]	71	7	15011	06xx				
3563	53/F	28 (Daughter)	80	45	16	01xx	05RV	3	3	4 (A,B,C,DR)	4 (A,B,C,DR)
			2	35	4	15xx	05RV				

*The mismatched haplotype for the patient is given on the top row and for the donor on the bottom row. Antigens or alleles in either the patient or donor that are homozygous, ie, present on the shared haplotype, are indicated by brackets.

†The locus (loci) of the mismatches are given in parentheses for cases involving fewer than all loci.

‡The first 5 patients and donors were typed by serology; all others were typed by SSP. Antigen equivalents are given for those typed by SSP.

§Class II alleles were defined by reverse SSOP or sequence-based typing, except for those alleles designated with "xx." In those cases, only low-resolution SSP typing was performed, as haplotypes were assigned from family genotyping.

||DQB1 alleles were typed to intermediate resolution by reverse SSOP and are designated by the codes of the National Marrow Donor Program.

Table 3. Outpatient Course*

Cohort	UPIN	Hematopoietic Recovery†		No. of Transfusions		No.‡	Length of Stay, d	Hospital Admissions
		ANC >500/μL	PLT >20,000/μL	RBC	PLT			Reason for Admission
1	3267	12	20	2	1	1	25	Day 42 fever/presumptive CMV infection
	3298	46	NR	15	15	2	3, 8	Day 3 neutropenic fever; day 10 fever/dehydration
	3303	NR	NR	21	38	2	5, 34	Day -1 neutropenic fever; day 24 fungal pneumonia
2	1706	16	22	2	1	1	4	Day 32 fever/sinusitis
	3384	13	12	1	1	1	2	Day 9 neutropenic fever
	3391	13	13	2	2	0		
	3434	15	0	8	0	1	4	Day 34 fever (drug-related)
	3271	15	26	7	5	0		
	3446	13	21	8	9	2	3, 6	Day 3 neutropenic fever; day 20 fever/swollen ankle joint
	3486	24	20	6	1	0		
	3529	44	146	14	19	1	3	Day 2 neutropenic fever
	3545	14	13	2	1	1	3	Day 3 neutropenic fever
	3563	15	14	3	1	2	4,8	Day 1 neutropenic fever; day 27 fever, diarrhea

*PLT indicates platelets; RBC, red blood cells; NR, no recovery.
 †Days post-HSCT.
 ‡Up to day +60, endpoint of study.

Outcomes: Cohort 1

These 3 patients received nonmyeloablative conditioning without pretransplantation Cy. The first patient (UPIN 3267), with secondary acute myeloid leukemia (AML) in first complete remission, experienced neutrophil recovery on day 12 (Table 3) but was found to be in hematologic relapse of leukemia on day 30 (Figure 2). Grade II acute GVHD of the skin and liver developed on day 60, following discontinuation of immunosuppression, but the patient died

of progressive disease on day 104. The remaining 2 patients did not achieve engraftment with donor cells, with 1 patient (UPIN 3298) exhibiting autologous neutrophil recovery on day 46 after BMT and the other (UPIN 3303) dying on day 58 of infection during aplasia. Both patients were mismatched with their donor grafts by 3 HLA antigens in the HVG direction, and serum taken from UPIN 3303 prior to transplantation was also found to contain lymphocytotoxic antibodies against donor HLA antigens.

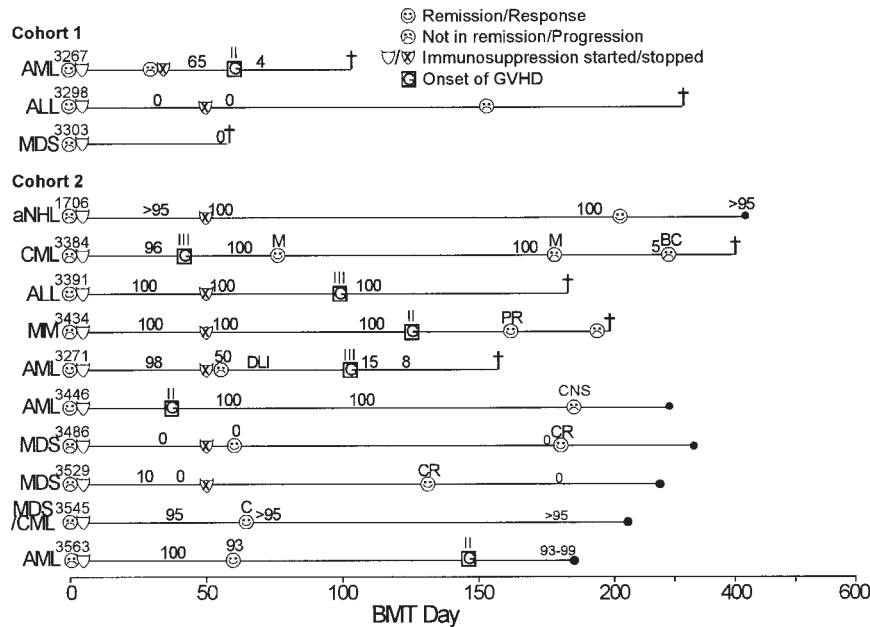


Figure 2. Patients' clinical courses. Diagnoses are listed to the left of each timeline. A Roman numeral above the GVHD symbol indicates maximum grade of GVHD. Numbers above day 0 are unique patient identifiers (UPIN); the numbers along the timeline are percentage donor chimerism. Disease states: M, molecular remission or relapse; BC, blast crisis; PR, partial response; CNS, central nervous system relapse; CR, complete response; C, cytogenetic remission; †, death.

Table 4. Chimerism, GVHD, and Patient Outcomes*

Cohort	UPIN	Diagnosis	HLA Mismatch†		Donor Chimerism, %			GVHD			Relapse, Day	Status, Day	
			HVG	GVH	Day ~30	Day ~60	Day ~180	Acute‡	Day	Chronic			
1	3267	AML	2 (A,B)	2 (B,DR)	70	23	ND	II (S)	52		30	Died§, 104	
	3298	Ph ⁺ ALL	3	3		0	ND				320	Died, 320	
	3303	MDS	3	1 (A)		0	ND					Died, aplasia, 58	
2	1706	NHL	1 (B)	1 (B)	100	100	100					Alive, NED, 423+	
	3384	CML-CP2	1 (A)	1 (A)	100	100	100	III (S,G)	42		271	Died, 401	
	3391	Ph ⁺ ALL	3	3	100	100	100	III (S,L)	100			Died, GVHD, 183	
	3434	MM	2 (A,DR)	2 (A,DR)	100	100	100	II (L)	126	Limited	111	Died, 198	
	3271	AML	3	2 (A,B)	>95	50	ND	III (G)	103		60	Died, 157	
	3446	AML	1 (DR)	1 (DR)	100	100	100	II (S)	38		183	Alive, in relapse 290+	
	3486	MDS/AML	2 (A,B)	3	0	0	0					Alive, NED 332+	
	3529	MDS	3	3	10	0	0					Alive, NED 277+	
	3545	MDS/CML	3	2 (B, DR)	>95	>95	>95						Alive, Cyto CR 225+
	3563	MDS/AML	3	3	>95	>95	93-99	II (S,G)					Alive, NED 185+

*ND indicates not determined; S, skin; NHL, non-Hodgkin's lymphoma; G, gastrointestinal; L, liver; MM, multiple myeloma; NED, no evidence of disease.

†n = number of mismatched antigens (mismatched loci)

‡Acute GVHD was defined as grades I-IV, maximum grade.

§Died in relapse unless otherwise specified.

Outcomes: Cohort 2

All 10 patients in this cohort received nonmyeloablative conditioning including pretransplantation Cy 14.5 mg/kg per day on days -6 and -5 (Figure 1).

Donor Chimerism. Sustained donor cell engraftment occurred in 8 of 10 patients in cohort 2 (Figure 2 and Table 4). In all 8 patients who had donor graft acceptance, donor chimerism was >90% on the first measurement. Chimerism subsequently remained >90% except in the 2 patients who developed bone marrow and hematologic relapse of their disease (UPIN 3384 and 3271). Failure of donor cell engraftment occurred in 0 of 3 donor-recipient pairs mismatched for 1 HLA antigen in the HVG direction, in 1 of 2 pairs mismatched for 2 HLA antigens, and in 1 of 5 pairs mismatched for 3 HLA antigens. Both patients who had graft rejection had a diagnosis of MDS, no prior chemotherapy, and a long history of transfusion dependence (Table 1).

Hematologic Recovery. Table 3 shows the neutrophil recovery of patients in cohort 2. All patients became neutropenic following conditioning therapy, with the white blood cell count nadir occurring at a median of day 9. Among the 8 patients who had donor graft acceptance, the median time to an absolute neutrophil count >500/μL was 15 days (range, 13-16 days), and the median time to platelet count >20,000/μL was 14 days (range, 0-26 days). Neutrophil recovery in the 2 patients with graft rejection occurred at days 24 and 44, and platelet recovery occurred by day 20 and 146, respectively. Among the patients with sustained donor cell engraftment, the median number of red blood cell transfusions was 3 (range, 1-8), and the median number of platelet transfusions was 1 (range, 0-9).

Hospital Admissions. As shown in Table 3, 10 patients were hospitalized for fevers, 7 of which occurred while the patients were neutropenic. The median number of admissions was 1 (range, 0-2), with a median hospital stay of 5 days (range, 2-34 days).

Acute GVHD. GVHD developed in 6 patients at a median of 99 days after BMT (range, 38-143 days; Figure 2 and Table 4). There were 3 cases of grade II GVHD and 3 cases of grade III GVHD. Of the 8 patients with sustained donor cell engraftment, GVHD occurred in 2 of 3 patients mismatched for 1 HLA antigen in the GVH direction, 2 of 3 patients mismatched for 2 HLA antigens, and both patients mismatched for 3 HLA antigens. Five patients responded to therapy, which included combinations of tacrolimus, steroids, psoralen-activated ultraviolet A light therapy, infliximab, daclizumab, or 2-deoxycoformycin. One patient died of infectious complications of GVHD.

In 3 of the patients, GVHD did not occur until day 98 or later, 48 or more days after discontinuation of immunosuppression. One of these patients (UPIN 3271) developed grade III GVHD 30 days after receiving a donor leukocyte infusion at a dose of 10⁵ CD3⁺ T-cells/kg for relapsed AML (Figure 2). Because of the delayed occurrence of GVHD, which was the proximate cause of death in 1 patient, the treatment protocol was modified to extend the duration of tacrolimus treatment to more than 90 days among patients with full donor hematopoietic chimerism at day 60. Since this modification was implemented, 2 patients have achieved engraftment with donor cells; 1 patient (UPIN 3563) developed grade II skin and gastrointestinal GVHD on day 143, which was controlled with a combination of steroids and 2-deoxycoformycin (Figure 2).

Immune Reconstitution. Table 5 shows recovery of NK cells and CD4 cells in the 6 patients of cohort 2 who were tested. With the exception of 2 patients, 1 of whom (UPIN 3384) was on high-dose corticosteroid therapy for acute GVHD, CD4 counts recovered to >200 cells/μL by day 40. NK cells recovered to pretransplantation levels by day 40 in all but 1 patient (UPIN 3434). In contrast, B-cell reconstitution was delayed (not shown). No detailed phenotypic analysis of NK cells or T-cells was performed.

Table 5. Lymphocyte Reconstitution in Cohort 2

UPIN	NK Cells/ μL		CD4 ⁺ Cells/ μL	
	Day -6	Day 40	Day -6	Day 40
1706	274	678	845	404
3384	86	228	716	69
3391	56	50	253	261
3434	166	50	417	366
3446	92	205	326	677
3486	121	156	887	146

Patient Outcomes. With a median follow-up of survivors of 284 days (range, 185-423 days), 6 of 10 patients are alive, and 5 patients are disease free (Table 4). Each of these 5 patients had active disease at the time of transplantation; therefore, each has responded to therapy. This group includes all 3 MDS patients, 2 of whom had graft rejection but remained in a complete remission 277 and 332 days after transplantation. The pretransplantation and most recent blood counts for these 3 patients are shown in Figure 3. Both of the patients who had graft rejection had a normal male karyotype in the bone marrow prior to transplantation; after transplantation, 1 patient (UPIN 3529) had a normal male karyotype and the other (UPIN 3486) had only nonclonal abnormalities in 9 of 30 cells analyzed. Flow cytometry of the marrow prior to transplantation revealed 22% phenotypically abnormal myeloid blasts in UPIN 3486 and 4% abnormal myeloid blasts in UPIN 3529; at 6 months after transplantation, no abnormal myeloid blasts were found in either of these patients. The pretransplantation marrow of the patient with MDS/CML (UPIN 3545) contained 4 clones, demonstrating trisomy 8 (4/22 cells), monosomy 7 (3/22 cells), both (6/22 cells), or only the Philadelphia chromosome (7/22 cells). Six months after transplantation, this patient's marrow contained >95% donor cells, a normal female karyotype, and only 1 of 6000 cells expressing a bcr/abl fusion signal by the FISH technique (normal, <5 cells).

GVHD did not appear to have a substantial beneficial effect on long-term disease-free survival, because the under-

lying malignancy either progressed or recurred in 4 of 5 patients who survived their GVHD. Three of the relapsing patients have died, and the fourth was treated with radiation therapy for a chloroma of the cerebrum. One patient died in remission from infectious complications of GVHD.

DISCUSSION

Although the combination of myeloablative conditioning and BMT from partially mismatched related donors has been used to treat hematologic malignancies in patients who lack an HLA-identical donor, this procedure carries a substantial risk of fatal graft rejection, GVHD, and serious infection resulting from prolonged immunosuppression. Rigorous depletion of T-cells from the donor graft reduces the risk of GVHD but increases the risk of graft rejection and prolongs posttransplantation immunosuppression [35-37]. GVH-reactive T-cells can be selectively tolerized by incubating the donor graft ex vivo with host cells in the presence of cytotoxic T-lymphocyte-associated antigen-4/immunoglobulin fusion protein (CTLA4-Ig), a recombinant fusion protein that blocks T-cell costimulatory interactions [38]. Although selective induction of tolerance in donor T-cells would in theory preserve donor T-cell responses to pathogens and promote faster immune reconstitution after BMT, the procedure does not obviate the need to condition the recipient intensively to prevent graft rejection. Thus, methods that promote selective tolerance induction in both host and donor T-cells, while preserving general immune responsiveness, are clearly needed.

Cy is a potent antineoplastic drug that has been extensively studied for its ability to suppress immune responses, including allogeneic reactions. Early studies demonstrated that Cy is most effective at suppressing MHC-incompatible skin allograft rejection when the drug is administered 2 to 3 days after placement of the graft [39,40]. Cy was also found to have the highest therapeutic ratio of all the cytotoxic agents tested for the promotion of immunologic tolerance [41]. In mouse models of MHC-identical allogeneic BMT, Cy can induce complete bidirectional tolerance, but only if the drug is given in a high dose, ≥ 150 mg/kg (300 mg/m²), 48 to 72 hours after transplantation [42]. We recently found

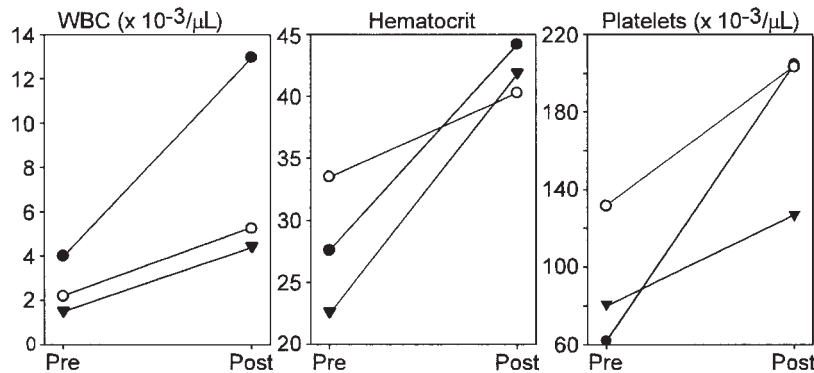


Figure 3. Pre- and posttransplantation blood counts from patients in cohort 2 with myelodysplasia. Pretransplantation counts were obtained on day -6; posttransplantation counts were taken on day 225 (○, UPIN 3545), day 277 (▲, UPIN 3529), and day 332 (●, UPIN 3486). Patients who had graft rejection are depicted by filled symbols.

that posttransplantation Cy significantly reduces both HVG and GVH reactions after MHC-mismatched BMT without impairing immune responses to unrelated third-party alloantigens [22]. The protocol of Cy-induced immunologic tolerance takes advantage of the drug's selective toxicity to recently activated lymphocytes. Thus, BMT stimulates the proliferation of both HVG- and GVH-reactive T-cells, which are selectively eliminated by the administration of Cy [43,44]. Although Cy, even in high doses, is not toxic to pluripotent hematopoietic stem cells [19,45], there was some concern that exposure of the donor graft to high-dose Cy would cause prolonged neutropenia. However, with filgrastim included in our protocol, this concern was not borne out clinically. The median time to a neutrophil count $>500/\mu\text{L}$ in this study was 15 days, which compares favorably to the median time to neutrophil recovery of 19 days in a retrospective study of patients receiving HLA-identical sibling bone marrow [46] and of 20 days among patients receiving T-cell-depleted marrow transplants from partially HLA-mismatched related donors [5]. These results suggest that high-dose posttransplantation Cy can be administered safely without substantially impairing hematologic recovery.

The major objective of this study was to determine the minimum dose of pretransplantation Cy that is required for the stable engraftment of partially HLA-mismatched marrow following nonmyeloablative conditioning including posttransplantation Cy. The risk of graft rejection following allogeneic BMT is influenced by a variety of factors, including conditioning regimen intensity, the chemotherapy and transfusion history of the recipient, donor/recipient histoincompatibility, donor CD34⁺ and T-cell dose, and posttransplantation pharmacologic immunosuppression. In a mouse model of MHC-mismatched BMT, we have found that a single high dose (200 mg/kg) of Cy is most effective at preventing graft rejection when given after, rather than before, the transplantation, but also that the engraftment-promoting effects of pre- and posttransplantation Cy are additive [22]. Because our patient group was quite heterogeneous with respect to most of these criteria, it is simply not possible to make firm conclusions about the relative importance of pre- versus posttransplantation Cy in preventing graft rejection. However, a number of conclusions about the effects of conditioning on transplantation outcome are possible.

First, because recovery of autologous hematopoiesis occurred in 3 of the 4 patients who had graft rejection, the conditioning regimens used in this study are truly nonmyeloablative. Three of the 4 patients who experienced graft rejection had a diagnosis of MDS, were transfusion dependent, and had not received any prior chemotherapy prior to the initiation of conditioning. In a separate study, patients with MDS were also found to have a higher risk of rejecting HLA-identical unrelated donor stem cells after nonmyeloablative conditioning than were patients with acute leukemia, lymphoma, or multiple myeloma [47]. It is not known whether the increased risk of graft rejection is due to an intrinsic immunologic abnormality associated with MDS or, as with aplastic anemia [48] and sickle cell anemia patients [49], due to sensitization by prior blood transfusions while immunocompetent. With regard to the latter possibility, we have found that conditioning with fludarabine, low-dose TBI, and posttransplantation Cy is insufficient to prevent

graft rejection in mice that have been previously sensitized to donor tissues (L.L., E.J.F., unpublished data).

Second, a clinical benefit of nonmyeloablative BMT can be obtained even in the absence of sustained donor cell engraftment, as illustrated by 2 patients with MDS (UPIN 3486 and 3529) who achieved a morphologic complete remission of their disease despite marrow graft rejection. This phenomenon has also been observed in patients with chemotherapy-refractory non-Hodgkin's lymphoma who lost donor chimerism following nonmyeloablative allogeneic stem cell transplantation [50]. There are at least 3 possible explanations for a therapeutic effect of nonmyeloablative allogeneic BMT in the absence of sustained donor cell engraftment. First, the direct cytotoxicity of fludarabine, TBI, and Cy on tumor cells may induce a remission in the absence of any immunologic anti-tumor effects. Second, a transient graft-versus-myelodysplasia effect, sufficient to eliminate the malignant cells, may occur before the donor graft is rejected. Finally, the BMT procedure may alter the immunologic milieu into one that is favorable to the activation of host-derived antitumor cytotoxic effector cells. In some rodent tumor models, Cy administration unmasks a therapeutic antitumor immune response [51] by eliminating CD4⁺ T-cells with suppressor activity [52] or by disrupting tumor stroma cell interactions [53]. Therefore, the immunologic effects of Cy administration in the conditioning regimen may not be limited to its effects on engraftment and GVHD.

Finally, the time to onset of GVHD in the patients treated here may be delayed compared to that of patients who receive haploidentical BMT after myeloablative conditioning. In one study that included 119 patients receiving 1 HLA antigen-mismatched BMT, nearly all patients who developed acute GVHD did so by day 30 after transplantation [4], whereas none of our patients had developed GVHD by that time. Several features of our protocol may contribute to a delayed onset and/or lessened severity of GVHD, including the use of reduced-dose TBI [54], posttransplantation Cy [22], and the combined administration of a calcineurin inhibitor (tacrolimus) and MMF for GVHD prophylaxis [24]. Of the 6 patients with GVHD, only 2 experienced the onset of disease while receiving tacrolimus and MMF, and donor chimerism in both of these patients was $>95\%$ before or shortly after disease onset (Figure 1). Because GVHD does not develop in the absence of full donor T-cell chimerism [55], we are now investigating the effect on the incidence and severity of GVHD of extending pharmacologic immunosuppression in full donor chimeras.

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