Partially Mismatched Pediatric Transplants With Allogeneic CD34+ Blood Cells From a Related Donor

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This was a phase I, multi-center study of 13 pediatric patients (median age, 11 years) to evaluate toxicity, hematopoietic recovery, and graft-versus-host disease (GVHD) after allogeneic transplantation of enriched blood CD34+ cells obtained from genotypically haploidentical but partially HLA-mismatched related donors (8 parents and 5 siblings). With regard to rejection, donor HLA disparity was 1 (5), 2 (6), or 3 loci (2). With regard to GVHD, recipient HLA disparity was 0 (1), 1 (3), 2 (8), or 3 (1). The patients suffered from acute myelogenous leukemia (6), chronic myelogenous leukemia (4), acute lymphoblastic leukemia (2), or hemolytic anemia plus immunodeficiency disorder (1). To reduce the risk of graft failure through the infusion of a large amount of stem cells, peripheral blood cells (PBC) were mobilized by recombinant granulocyte colony-stimulating factor (G-CSF; lenograstim, 10 μg/kg/d for 5 days) and collected by 2 to 5 aphereses. To both enhance engraftment and reduce GVHD, CD34+ cells were enriched using immunomagnetic procedures with the Baxter ISOLEX 300 system (Baxter Healthcare Corp, Irvine, CA) and cryopreserved. After variable cytoreductive regimens, a median of 7.7 (range, 2.2 to 14) × 10^6/kg of CD34+ cells and 1.03 (0.05 to 2.09) × 10^5/kg CD3+ cells were infused. Using Center-specific posttransplant supportive care and immunosuppressive GVHD prophylaxis, two patients experienced early death; one from veno-occlusive disease at day 17 and one from sepsis at day 18. Nine of 11 patients showed signs of engraftment; however, subsequent rejection was seen in 4 patients, 2 of whom had autologous recovery. Eight patients were evaluated in the early phase of marrow recovery. The median number of days to achieve an absolute granulocyte count of 0.5 × 10^9/L was 14 (range, 9 to 20) and that to achieve a platelet count of 20 × 10^9/L was 17.5 (range, 12 to 23). Donor chimerism persisted in five patients until death or current survival. All of the surviving patients with functioning-donor–type hematopoiesis were given total body irradiation. De novo acute GVHD (grades II and IV) was observed in two of the eight evaluated patients. Scheduled donor lymphocyte infusion (DLI), using the CD34− fraction, was administered to four patients, free of de novo acute GVHD, beginning between 28 to 43 days after transplant. Three of these patients developed acute GVHD (grades I, II, and IV). Cytomegalovirus infection was a major infectious complication but was successfully managed with γ-globulin and gancyclovir treatment with or without additional DLI. Five patients are currently surviving, free of disease, with a follow-up ranging from 476 to 937 days. Each survivor has functioning hematopoiesis, three of donor origin and two of autologous origin. In conclusion, our results show that enriched blood CD34+ cells from a mismatched haploidentical donor are a feasible alternative source of stem cells, but do not appear to ensure engraftment. Because none of the patients who were administered DLI survived, the therapeutic efficacy and safety of periodic DLI, as an integrated part of such transplants, needs to be clarified in further studies.

ONE MAJOR LIMITATION of allogeneic bone marrow transplantation (BMT) is the lack of suitable donors. The results of transplantation with unrelated marrow grafts are, in general, inferior to those using matched sibling marrow, with an increased incidence and severity of graft-versus-host disease (GVHD), graft rejection, and infections,[1-4] although new immunosuppressive therapies have made the prophylaxis or treatment of GVHD more effective. In addition, the search for an unrelated matched donor is time-consuming, and rapid disease progression in some patients makes this approach impractical. Alternative procedures include transplanting cells from a related mismatched donor or cord blood cells. The frequency of serious GVHD is reported to be far lower in transplants with readily available cord blood cells, but this procedure is limited by rejection or slow engraftment, possibly due to the small content of stem cells in the graft.[5]

The use of an HLA-mismatched related donor avoids the lengthy search procedure and provides donors for greater than 90% of patients who may potentially benefit from allogeneic transplantation.[6] Depletion of T lymphocytes (T cells) from the graft can reduce the number of T cells to below the critical threshold needed for the development of severe de novo GVHD, and this has proved to be the most effective method for preventing acute GVHD in BMT.[7] However, this procedure is associated with an

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increased incidence of graft rejection, as well as with an increase in leukemia relapse and B-cell lymphoproliferative disorders (BLPD).

Mobilized allogeneic peripheral blood cells (PBC) have recently been shown to be a potential source of stem cells for hematopoietic reconstitution after myeloablative therapy.[8,9] Most importantly, more hematopoietic cells can be collected from blood than from BM or cord/placenta. Nevertheless, substantially more T cells than BM are in PBC grafts, which may lead to a higher risk of de novo GVHD. In a previous study, we showed that negative depletion of T cells is unsatisfactory with PBC grafts, and is associated with a significant loss of hematopoietic progenitor cells.[10] On the other hand, recently developed techniques for the positive selection/enrichment of $CD34^+$ cells from marrow or PBC provide a convenient method for concentrating hematopoietic progenitors and depleting T cells.[11]

The present phase I study of 13 children was designed to examine the safety and effectiveness of blood $CD34^+$ cells enriched from genotypically haploidentical but partially mismatched related donors. To prevent serious cytomegalovirus (CMV) infection and BLPD, and to induce a graft-versus-leukemia (GVL) effect, patients who did not develop de novo GVHD by day 28 were scheduled to receive periodic infusion of an escalating dose of thawed donor lymphocytes contained in $CD34^-$ cells, starting from 1×10^5/kg. The primary goal of this study was to examine whether this new approach could provide a safe and effective graft while avoiding serious GVHD. We believe that the results of this small trial may be useful for the development of strategies for the clinical application of enriched $CD34^+$ cells.

MATERIALS AND METHODS

Human subjects and histocompatibility studies. Patients who were currently eligible for allogeneic transplant were enrolled into the study. All of the patients lacked an HLA-identical sibling or unrelated donor, but did have a readily available healthy donor within the family who met the donation criteria. In preparation for the donation, the donor underwent a detailed medical history and physical examination according to the institution's standard procedures.

A total of 13 patients, 9 male and 4 female, were treated in participating institutes with the approval of the respective institutional review boards. The patients ranged in age from 1 to 18 years, with a median of 11 years, and their clinical characteristics are shown in Table 1. The diagnosis included acute myelogenous leukemia (AML 6; M1 3, M4 1, M5 2), chronic myelocytic leukemia (CML 4; 2 in the chronic phase and 2 at blastic crisis), acute lymphoblastic leukemia (ALL, 2), and congenital immunodeficiency disorder associated with intractable hemolytic anemia (1). The corresponding donors ranged from 5 to 43 years old (median, 35). HLA-A and -B were typed by serological tests and DRB1 typing was performed using DNA-based techniques. Mixed lymphocyte culture was not performed for each patient/donor pair. With regard to rejection, donor HLA disparity was 1 (5), 2 (6) or 3 loci (2). With regard to GVHD, recipient HLA disparity was 0 (1), 1 (3), 2 (8), or 3 (1).

The administration of human recombinant granulocyte colony-stimulating factor (G-CSF; lenograstim, Chugai Pharmaceutical Co, Tokyo, Japan) to healthy donors, collection of PBC, and subsequent transplants with enriched $CD34^+$ cells were all approved by the institutional review boards of the participating hospitals. Written informed consent was obtained from all of the patients and donors or their guardians.

Mobilization and apheresis of PBC. All cell-preparation procedures, including mobilization, $CD34^+$ enrichment, and cryopreservation, were performed at the University of Tokushima as previously published.[12] The donors received G-CSF 5 μg/kg/twice a day, or 10 μg/kg/once a day by subcutaneous injection for 5 days.[13] Apheresis was initiated from day 4 to 6 after G-CSF injection, and 300 mL/kg (maximum, 10 L) were processed per session.[12] Apheresis was principally continued until a total of $>6 \times 10^6$ $CD34^+$ cells/kg recipient weight were collected. The details of the procedure for collecting PBC using the Fenwal CS 3000 plus (Baxter Limited, Tokyo, Japan) combined with a small volume collection chamber have been described previously.[12]

Isolation and cryopreservation of $CD34^+$ cells. PBC collected by apheresis were enriched for $CD34^+$ cells using the ISOLEX-300 system (Baxter Healthcare Corp, Irvine, CA) according to the manufacturer's suggestions. Briefly, excess platelets were removed by centrifugation for 20 minutes at 200*g* at room temperature. Cells were incubated in phosphate-buffered saline (PBS; Nissui, Tokyo, Japan) containing 0.5% human α-globulin (Gammagard; Baxter, Tokyo, Japan) for 15 minutes to block Fc-receptors. One vial of anti-CD34 monoclonal antibody (MoAb) (9C5, 2 mg) was added to the cell suspension which contained $< 5 \times 10^{10}$ cells. After 30 minutes of incubation at room temperature with gentle rotation (4/min), cells were washed three times with PBS containing 1% human serum albumin (Albumin-Midori; Green Cross

Table 1. Characteristics of Donors and Patients

Patients						Donors			HLA-Disparity	
Case	Diagnosis	Sex	Age	Weight (kg)	Status	Relation	Age	Weight (kg)	Rejection	GVHD
1	AML(M1)	F	11	40	2nd relapse, refractory	Father	43	80	2	2
2	HA, ID	F	1	8.5	Refractory	Sister	5	18	2	2
3	ALL	F	11	24	2nd relapse, 3rd CR	Brother	13	50	3	2
4	AML(M1)	M	14	50	1st relapse, refractory	Sister	18	50	1	2
5	CML	M	10	44	Ph^+, adult type	Father	40	70	2	2
6	AML(M1)	M	14	42	Induction failure	Father	42	62	1	0
7	ALL	F	10	46	Relapse after auto-PBSCT	Sister	14	76	1	1
8	CML	F	12	49	Ph^+, adult type	Father	41	69	2	2
9	CML	M	10	30	Blastic phase, uncontrollable	Mother	35	50	1	1
10	AML(M5)	M	2	12.7	Very early relapse	Father	36	54	1	1
11	CML	M	18	59	Ph^+ CML, blastic phase	Brother	20	73	3	3
12	AML(M5)	M	1	8.5	1st CR, infant leukemia	Mother	29	51	2	2
13	AML(M4)	M	16	81	Secondary leukemia, refractory	Mother	43	54	2	2

Abbreviations: HA, hemolytic anemia; ID, immunodeficiency; CR, complete remission.

Co, Kyoto, Japan). Sensitized cells were incubated with sheep anti-mouse IgG_1-coated paramagnetic microspheres (Dynabeads; Dynal, Oslo, Norway; 10 mL). Cells rosetted with beads were captured on permanent magnets and released by chymopapain or peptide capture included in the kit.

The enriched $CD34^+$ cells were cryopreserved using the hydroxyethyl starch (HES)/dimethyl sulphoxide (DMSO) method without controlled-rate cooling.[14] Briefly, $CD34^+$ cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% autologous serum. Cells were slowly mixed with an equal volume of a cryoprotectant solution containing 8% human albumin, 10% DMSO, and 12% HES to give final concentrations of 5% DMSO and 6% HES. Cells were then transferred to 5-mL polypropylene tubes and placed directly in an electric freezer that maintained a temperature of −135°C. The non-CD34 cell fraction was also cryopreserved in tubes as a T-cell source for donor lymphocyte infusion (DLI) in selected patients.

Hematopoietic progenitor assay and flow cytometry analysis. The details of the hematopoietic progenitor assays have been reported elsewhere.[15] Briefly, cells were incubated in methylcellulose medium supplemented with 20% fetal bovine serum (FBS), 450 μg/mL of human transferrin (T-1145; Sigma Chemical Co, St Louis, MO), 2 U/mL of human recombinant erythropoietin, 1% crystallized bovine serum albumin (Calbiochem 12657; Hoechst Japan, Tokyo), interleukin-3 (IL-3; 20 ng/mL; Kirin Brewery Co, Tokyo, Japan), stem cell factor (20 ng/mL; Kirin Brewery Co), and G-CSF (20 ng/mL). Cells were placed in 24-well culture plates (Corning 258201; Corning, NY) in quadruplicate, and incubated in an ESPEC N_2-O_2-CO_2 BNP-110 incubator (Tabai ESPEC Co, Osaka, Japan) which maintained a humid atmosphere of 5% carbon dioxide, 5% oxygen, and 90% nitrogen. After 14 days of incubation, colony-forming units for granulocyte-macrophage (CFU-GM) were scored using an inverted microscope.

Cells expressing the surface CD34 antigen were identified by flow cytometry analysis as previously reported.[16] One hundred microliters of cell suspension were added to a test tube (Falcon 2052; Becton Dickinson, Lincoln Park, NJ) containing isotype control (phycoerythrin-mouse IgG_1) and phycoerythrin-conjugated CD34 MoAb (Anti-HPCA2 antibody; Becton Dickinson) at a concentration of 1 μg antibody/10^6 cells. After 30 minutes of incubation in the dark, cells were washed twice and resuspended in PBS containing 1% bovine serum albumin (BSA). Red blood cells in the sample were lysed with a solution of 0.826% (wt/vol) NH_4Cl, 0.1% $KHCO_3$, and 0.004% EDTA-4Na. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson). A total of 20,000 events were counted to identify the mononuclear cell (MNC) fraction. The flow cytometric data were analyzed using a gated analysis via a set of SSMC-FL parameters for $CD34^+$ cells to calculate the percentage of positive cells. $CD3^+$ cells were also analyzed using specific MoAb (OKT3; Ortho, Raritan, NJ).

High-dose chemotherapy and transplant procedures. All of the patients were treated in laminar air flow rooms and administered oral nonabsorbable antibiotics. Cytoreductive regimens and immunosuppressive agents are summarized in Table 2. Enriched $CD34^+$ cells were thawed and infused into the recipient without manipulation. Prophylaxis against GVHD included cyclosporine A (CyA) beginning on day 0, at a daily dose of 5 mg/kg (10 patients). Conversion to oral CyA was deferred until signs of gastrointestinal toxicity subsided. Seven of the 10 patients were also treated with 10 mg/m^2 of methotrexate (MTX) on days 1, 3, and 6. The other two patients received tacrolimus (FK506) or

Table 2. Transplant Procedures and Engraftment Data

			Infused Cells				Engraftment (d)		
								PLT	
Case	Cytoreductive Regimens	GVHD Prophylaxis	CD34 ($\times 10^6$/kg)	CFU-GM ($\times 10^5$/kg)	CD3 ($\times 10^5$/kg)	Use of G-CSF	AGC (0.5×10^9/L)	(>20 × 10^9/L)	(>50 × 10^9/L)
1	L-PAM (210 mg/m²), BU (16 mg/kg), OKT3	MPSL	9.7	19.4	1.75	No	11	(−)	(−)
2	TBI (7.5 Gy), ATG (10 mg/kg), CY (200 mg/kg)	CyA + short-term MTX	3.9	11.7	0.39	Yes	12	18	19
3	TBI (12 Gy), ATG (10 mg/kg), L-PAM (140 mg/m²), TESPA (600 mg/m²)	CyA + short-term MTX	4.1	4.5	2.09	Yes	(−)	(−)	(−)
4	TBI (10 Gy), AraC (100 mg/m²) cont, HD-AraC (12 g/m²), CY (120 mg/kg), ATG (10 mg/kg)	CyA	6.4	5.4	1.07	Yes	16	23	26
5	BU (16 mg/kg), AraC (18 g/m²), CY (100 mg/kg)	CyA + short-term MTX	5.9	17.5	0.73	Yes	20	17	(−)
6	TBI (12 Gy), CY (120 mg/kg), TESPA (10 mg/kg)	FK506	14	22.3	1.03	Yes	14	14	36
7	TBI (12 Gy), VP-16 (60 mg/kg), L-PAM (180 mg/m²)	CyA + short-term MTX	9.6	3.8	0.02	Yes	9	14	15
8	TBI (12 Gy), CY (200 mg/kg)	CyA + short-term MTX	7.7	1.5	ND	Yes	(−)	(−)	(−)
9	TLI (10 Gy), L-PAM (210 mg/m²), BU (600 mg/m²)	CyA + MPSL	4.7	8.8	0.58	No	9	12	12
10	BU (16 mg/kg), VP-16 (50 mg/kg), L-PAM (140 mg/m²)	CyA + short-term MTX	11.5	16	0.05	Yes	(−)	(−)	(−)
11	TBI (4 Gy), ALG (1,500 mg), CY (60 mg/kg)	CyA + MPSL	3.4	6.8	0.65	Yes	16	22	25
12	AraC (12 g/m²), VP-16 (1,600 mg/m²), CY (100 mg/kg)	CyA + short-term MTX	7.9	0.7	2.01	Yes	(−)	(−)	(−)
13	BU (16 mg/kg), L-PAM (210 mg/m²), TLI (6 Gy)	None	2.2	4.9	1.3	Yes	19	18	(−)

Abbreviations: L-PAM, melphalan; BU, busulfan; CY, clopphosphamide; TESPA, thio-tepa; AraC, cytosine arabinoside; ND, not determined; TLI, total lymphoid irradiation.

methylprednisolone (MPSL) instead of CyA with or without MTX. One patient was not administered prophylactic immunosuppressants. Eleven patients received G-CSF after transplantation. Support given to the patients included transfusions of blood products, antimicrobial drugs, nutritional support, and other measures as determined by clinical conditions and institutional protocol, and according to the accepted standards of medical care.

To prevent serious viral infection and BLPD, and/or to induce a GVL effect, patients who did not develop de novo GVHD by day 28 were scheduled to receive a weekly infusion of an escalating dose of cryopreserved/thawed donor $CD34^-$ cells containing lymphocytes, starting at 1×10^5/kg in selected institutes. No attempt was made to further manipulate the $CD34^-$ fraction. The end point was either development of GVHD or six weekly infusions.

Assessment of engraftment and GVHD. The early phase of engraftment was defined as trilineage transfusion-independent recovery of normal peripheral blood cell counts. Neutrophil engraftment day was defined as the third consecutive day on which the absolute granulocyte count (AGC) exceeded 0.5×10^9/L. Platelet engraftment day was defined as the third consecutive day on which the count exceeded 20 or 50×10^9/L. Sustained and long-term engraftment was evaluated by cytogenetic markers or by a DNA analysis for variable number tandem repeats (VNTR). The severity of acute GVHD was graded according to the Seattle score.[17]

RESULTS

Donor response to G-CSF administration. Although some suffered from a flulike syndrome including mild lumbago, headache, and fatigue, all of the donors generally tolerated the mobilization regimen well and none were removed from the protocol. In all of the donors, the white blood cell (WBC) count started to increase on the second day of G-CSF injection, and reached a peak value of 45 (range, 21 to 57) $\times 10^9$/L on day 5. The percentage of $CD34^+$ cells in peripheral MNC was less than 0.15% in all of the donors before G-CSF treatment, and this increased to 1.17% (range, 0.04% to 3.08%) on day 4 or 5. The donors then underwent an average of 3 aphereses (range, 2 to 5), with no immediate side effects directly related to the procedure. The numbers of MNC, CFU-GM, and $CD34^+$ cells collected are shown in Table 3. The target number of $CD34^+$ cells was enriched in all but two donors (cases 9 and 13) who had $<2 \times 10^6$/kg $CD34^+$ cells/kg after enrichment. These

Table 3. Cell Purification Data

		Cells in the Bags				$CD34^+$ Cell Fraction				
						$CD34^+$ Cells			CFU-GM	
Case	No. of Purification	MNC ($\times 10^{10}$)	% of $CD34^+$ Cells	$CD34^+$ Cells ($\times 10^8$)	CFU-GM ($\times 10^5$)	Purity (%)	Yield (%)	$\times 10^6$	Yield (%)	$\times 10^5$
1	1	10.4	1.78	18.51	2308	50.5	25.6	227	35.1	810
	2	3.1	0.90	2.79	1300	80.9	44.4	124	20	260
2	1	1.2	0.45	0.54	915	18.8	14.6	256	8.2	75
	2	3.2	0.82	2.62	750	72.6	10.8	54	4.6	34.5
3	1	4.8	1.64	7.87	ND	22.4	6.4	241	ND	110
4	1	2.6	0.77	2.00	456	84.5	64.8	127	21.5	98
	2	7.8	0.92	7.18	1167	77.0	26.7	192	15	175
5	1	1.2	0.69	0.83	180	94.8	104	85	97.8	176
	2	4	0.89	3.56	738	97	48	175	80.5	594
6	1	3.4	0.82	2.79	595	89.8	80.3	224	50.4	300
	2	4.5	1.05	4.73	1434	83	77	365	44.5	638
7	1	9.9	0.65	6.45	145	97.4	28	181	ND	ND
	2	5.9	0.63	3.72	33	88.2	70.5	262	ND	ND
8	1	6.6	1.03	6.88	ND	94	44.3	305	ND	ND
	2	4.6	0.57	2.63	ND	93	25.1	66	ND	ND
	3	3.5	0.66	2.30	ND	97.9	56.3	116	ND	ND
	4	3.4	0.8	2.70	ND	94.6	28.9	78	ND	ND
	5	3.4	0.65	2.22	ND	95.2	39.6	88	ND	ND
9	1	2.3	0.27	0.35	136	78.7	43.6	27.5	50	68
	2	1.4	0.48	0.5	384	77.4	57.6	38.7	25.5	98
	3	4	0.54	2.17	636	94.5	36.9	84	3.3	21
10	1	6.1	0.89	5.45	813	84	24.6	134	21.6	176
	2	1.6	0.68	1.09	40	65.8	12.1	13.2	72.5	29
11	1	4.3	0.41	1.74	675	22.1	8	14	3.3	22
	2	5.1	0.79	4.04	1560	73.5	36.4	147	24.4	380
12	1	5.5	0.77	3.95	40	55.7	16.9	66.8	15	6
13	1	4.1	0.55	2.25	465	53.7	16.4	37	65.7	148
	2	1.71	0.10	0.17	100	77	112	19	36	36
	3	1.53	1.00	1.53	311	63.3	26.8	41	48.2	150
	4	1.89	1.06	2.01	627	62.5	40.4	81.3	31.1	195
Mean		4.10	0.78	3.52	659	74.7	40.9	129	35.2	200
SD		2.36	0.34	3.51	568	22.7	27.1	94	26.1	215

Abbreviation: ND, not determined.

donors underwent a second mobilization by G-CSF 2 weeks later, and the combined number of $CD34^+$ cells then exceeded 2×10^6/kg.

Enrichment of $CD34^+$ cells. A total of 30 procedures were performed. The entire procedure, after apheresis to cryopreservation, was completed within 5 hours and the enrichment results are summarized in Table 3. The median number of cells subjected to enrichment was 3.8 (range, 1.2 to 10.4) $\times 10^{10}$, and 2.63 (range, 0.14 to 3.65) $\times 10^8$ $CD34^+$ cells were recovered with a purity of 80% (range, 19% to 98%). The median yield of $CD34^+$ cells and CFU-GM was 37% and 28%, respectively. Consequently, an average of 7.0 (range, 2.2 to 14) $\times 10^6$/kg $CD34^+$ cells and 0.97 (range, 0.05 to 2.09) $\times 10^5$/kg $CD3^+$ cells were infused (Table 2).

Regimen-related toxicity (RRT) and engraftment. Engraftment results are shown in Table 2. On the 28th day after transplantation, sustained engraftment was examined by cytogenetic markers or DNA analysis. With regard to early engraftment, one patient (case 1) who underwent transplant at refractory relapse of AML with 9.7 $\times 10^6$/kg $CD34^+$ cells failed to engraft after a transient increase in the neutrophil count to 1.1 $\times$ 10^9/L. This patient died of RRT on day 43. Case 3 died of hepatic veno-occlusive disease (VOD) on day 17 and case 12 died of complicating sepsis at day 18. Two other patients (cases 8 and 10) developed autologous recovery of hematopoiesis without a transient increase in donor-type neutrophils and are currently alive, with no evidence of malignant disease.

Hence, cases 1, 3, 8, 10, and 12 were considered failed engraftment, and were excluded from the analysis of early engraftment. The median number of days to achieve an AGC of 0.5 $\times 10^9$/L and platelet counts of 20 $\times 10^9$/L and 50 $\times 10^9$/L was, respectively, 14 (range, 9 to 20, n = 9), 17.5 (range, 12 to 23, n = 8), and 22 (range, 12 to 36, n = 6). Case 11 developed late rejection of the graft after transient recovery of hematopoiesis and subsequently developed functioning autologous recovery, but died of recurrent leukemia.

GVHD and clinical course. De novo acute GVHD (grades II and III) was observed in 2 (cases 6 and 7) of the 9 evaluated patients (Table 4). These patients received cells from matched and one-loci mismatched donors with regard to GVHD, containing 1.03 and 0.02 $\times 10^5$/kg $CD3^+$ cells, respectively. Both could be managed without the additional use of immunosuppressants. Consequently, the addition of T cells to the $CD34^-$ fraction was tested in 4 of the 8 eligible patients. Two of these 4 patients (cases 4 and 5) developed grade II and IV acute GVHD, respectively, after a single course of DLI. Although both were successfully managed with additional immunosuppression, case 4 developed recurrence of leukemia and eventually died on day 277, and case 5 died of sepsis before achieving a platelet level of 50 $\times 10^9$/L on day 116.

One of the 4 patients (case 9) who received DLI showed 100% donor-type reconstitution on 28 day after transplantation. Four courses of weekly DLI therapy were suspended when the patient developed grade I GVHD. Four weeks later, DLI therapy was resumed when the patient was found to be a chimera (87%) and CMV-antigenemia$^+$ with a titer of $>10/5 \times$ 10^4 cells; both conditions promptly resolved after two courses of DLI. Because this patient developed pancytopenia 10 months after transplantation, we reinfused 6.3 $\times 10^8$/kg of MNC in $CD34^-$ fraction. As a result of this procedure, he developed acute GVHD combined with pneumonitis and multi-organ failure, and died on day 363 despite supportive therapies.

The major infectious complication was cytomegalovirus infection, which was observed in three patients. All events were promptly resolved with γ-globulin and gancyclovir treatment with or without additional DLI. Currently, five patients are surviving with a follow-up ranging from 476 to 937 days; all of these patients, including two cases with autologous marrow recovery (cases 8 and 10), are completely free of disease. Case 8 with CML is in complete remission without any sign of Ph^+ cells. None of these patients has developed BLPD or chronic GVHD.

DISCUSSION

The primary cause of mortality in transplantation with a mismatched donor is engraftment failure due to graft rejection

Table 4. Complications and Outcome

Case	Primary Acute GVHD	Complications	DLI		GVHD After DLI	Treatment of GVHD in Addition to Prophylaxis	Current Status	Cause of Death (d)
			Starting Day	Times/Total Dose of MNC (×10⁶/kg)				
1	None	Sepsis, mucositis	(−)	—	(−)	(−)	Dead	Rejection, RRT (d 43)
2	None	None	(−)	—	(−)	(−)	Alive 937+	
3	NE	VOD, renal failure, sepsis	(−)	—	(−)	(−)	Dead	VOD (d 17)
4	None*	None	34	5/1.4	II	MPSL	Dead	Relapse (d 277)
5	None*	Convulsion	43	1/10	IV	MPSL, FK506	Dead	GVHD, sepsis (d 116)
6	II(d10−)	CMV cystitis	(−)	—	(−)	(−)	Alive 754+	
7	III(d17−)	Convulsion	(−)	—	(−)	(−)	Alive 651+	
8	NE	Sepsis, pyramidal sign	(−)	—	(−)	(−)	Alive 594+	Autologous recovery (Ph+ free)
9	None	None	30	28/1204	IV	MPSL	Dead	Peumonitis, MOF (d 363)
10	NE	None	(−)	—	(−)	(−)	Alive 476+	Autologous recovery
11	None	None	(−)	—	(−)	(−)	Dead	Rejection, relapse (d 262)
12	NE	Sepsis, DIC	(−)	—	(−)	(−)	Dead	Sepsis (d 18)
13	None*	Mucositis	43	3/20.5	I	None	Dead	Relapse (d 59)

Abbreviations: NE, not evaluable; VOD, veno-occlusive disease; FK506, tacrolimus; MOF, multi-organ failure.
*GVHD developed after DLI.

mediated by residual host T cells resistant to conventional conditioning regimens, which heavily depend on the degree of donor incompatibility.[18] To reduce the rate of graft failure by eliminating residual T cells, cytoreductive conditioning regimens can be intensified by increasing the total dose of total body irradiation (TBI) or by intensifying immunosuppression thereafter.[19] However, increasing the intensity of TBI usually leads to increased RRT.[20] The other primary problem in this setting is the occurrence of GVHD, which is caused by contaminating T cells in the graft. To prevent this, various strategies have been attempted with marrow cells. In one study with pediatric patients, BMT with a mismatched family donor was associated with a decreased incidence of serious GVHD compared with historical cohorts when ex vivo and in vitro T-cell depletion by MoAb was applied.[6,21] An alternative approach includes the indirect depletion of T cells by isolating $CD34^+$ cells. Additional advantages of the enrichment of $CD34^+$ cells include a reduction in the volume of cells which may lead to toxic complications at infusion.[22] Removal of contaminating erythrocytes may also be important for preventing hemolysis at thawing, which may cause acute renal failure, or for ABO-mismatched grafts between donor and patient. However, this procedure is associated with an increased incidence of graft rejection, and an increase in leukemia relapse caused by the lack of a GVL effect and BLPD.

In this study, we intended to reduce the incidence of graft rejection by increasing the stem cell dose, which can be achieved using mobilized PBC. This was based on the idea that standard marrow harvest from the iliac crests results in a suboptimal cell dose for T-cell–depleted transplants and causes delayed engraftment. Mobilized PBC have been widely used for allogeneic transplantation. Although ethical concerns, including oncogenesis, still exist regarding the use of G-CSF for normal volunteer donors, we believe that the risk of marrow aspiration under general anesthesia still exceeds that associated with G-CSF.[23]

The primary endpoint of this study with a small number of patients was feasibility. Although this study was contaminated by an inevitable bias caused by the variation in conditioning and immunosuppressive regimens, which may affect our assessment of the development of graft rejection and GVHD, the data we obtained are still informative. To avoid long-term toxicities in growing children, half of our patients did not receive a TBI-containing cytoreductive regimen. However, the primary cause of mortality in this study was a high incidence of graft rejection, even though there were only two haploidentical-mismatched transplants with regard to rejection and we infused a median of 7×10^6/kg $CD34^+$ cells; one patient did not engraft at all and, despite a rapid early recovery of hematopoiesis, four patients subsequently rejected the graft. All of the surviving patients with functioning donor-type hematopoiesis had been treated by TBI, while engraftment failed in most of those who did not receive TBI.

Henslee-Downey et al[24] recently published an update of their pioneering work in transplantation in mismatched family pairs. Seventy-two uniformly treated patients who underwent allogeneic transplant with T-cell–depleted BM obtained from haploidentical related family members were conditioned with a regimen containing TBI with follow-up immunosuppression. The median number of $CD34^+$ cells infused was far lower than 1.36×10^6/kg, but the overall probability of engraftment was 88% at 32 days, although this decreased to below 70% in three-antigen rejection mismatched pairs. They suggested that more intense immunosuppression by the use of a higher dose of TBI is crucial for supporting engraftment, and this appears to have been confirmed in our current study. Bacigalupo et al[25] reported their experience in 10 patients using a combination of $CD34^+$ cells purified from BM and G-CSF–primed peripheral blood in HLA-mismatched pairs. After conditioning with TBI and ALG, the patients received a median of 5.7×10^6/kg $CD34^+$ cells, and all showed successful engraftment. Considering all of these results, it appears as though the content of $CD34^+$ cells in the graft is not the sole factor that ensures stable engraftment after this mode of transplantation. It has also been reported that the presence of an optimal dose of T cells in the graft is another primary factor that ensures stable engraftment.[24,26] Because the development of significant GVHD was not a major obstacle in our study, it is possible that our procedure removed most of the accessory cell populations, which are responsible for GVHD and/or for facilitating engraftment. In the application of a negative purging system to BM cells,[24] it is probable that only accessory cells responsible for GVHD were removed without the loss of veto cell activities, which would thereby facilitate engraftment with a far smaller number of $CD34^+$ cells.

In contrast to our results, the primary problem in previous studies on transplantation with isolated $CD34^+$ cells has been the occurrence of GVHD, even in HLA-identical siblings.[27] Most of the patients treated by Bacigalupo et al developed acute GVHD greater than grade II while on GVHD prophylaxis with CyA after conditioning with TBI and ALG.[25] The incidence of acute GVHD in the report by Laport et al,[28] who used prophylaxis consisting of CyA with or without MTX, was 42% for grade I-II and 21% for grade III-IV. Henslee-Downey et al[24] reported that a greater number of T cells in the graft was associated with a higher risk of severe acute GVHD and that the timing of transplant was the most important factor in improving long-term survival. In their study, chronic GVHD occurred in 35% of evaluable patients. On the other hand, in our series, de novo acute GVHD occurred in two patients. Clinically significant GVHD developed only after the initiation of scheduled lymphocyte infusion. Thus, our procedure for the purification of $CD34^+$ blood was successful in terms of T-cell depletion as a primary endpoint.

Avoidance of clinically significant GVHD, while retaining a GVL effect, engraftment, and antiviral potential, is of prime importance. Patients who show stable mixed chimerism after BMT may benefit from allogeneic cell therapy with immunocompetent lymphocytes and stem cells.[29] Consequently, we added back a low number of cryopreserved T cells contained in the $CD34^-$ fraction after achieving stable engraftment, as has been tried clinically in BMT.[30] GVHD may be more likely to occur when the cells are infused in the initial allograft than if the infusion is delayed until after initial engraftment and hematological recovery. Although complications including sepsis or viremia due to profound immunodeficiency have been the primary cause of death in many patients who have undergone allogeneic transplantation with selected $CD34^+$ cells,[25,28] we encountered

few such complications. Although the early application of periodic DLI may become useful for preventing both late graft rejection and CMV infection, the optimum dose and time schedule of DLI have not yet been established.

In conclusion, although our findings must be viewed as preliminary given the small number of children and short follow-up period, we showed that at least some groups of children at very high risk of disease without a matched donor may benefit from our procedure. This gives us the rationale to continue our feasibility study, because our approach may provide a readily available donor for most candidates for allogeneic transplant. The incidence of chronic GVHD and the long-term stability of hematopoiesis will be determined in a longer follow-up of a larger number of patients.

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