Table 2 characteristics of AdV disease

Patient no.	*GVHD*	*Immunosuppressive at HC onset*	*Serotype of AdV*	*Viral study at the onset of HC culture/ PCR/IC*	*Onset of HC (days from transplant)*	*Start of CDV administration (days from transplant)*	*Interval from onset to CDV treatment (days)*	*Prior therapy for HC*
1	Grade II	FK506/PSL	11	+/+/+	40	59	19	AraA
3	No	Cs	11	+/+/+	29	30	1	No
4	NE	PSL	11	+/+/+	63	66	3	No
5	No	FK506/PSL	35	+/+/+	17	17	0	No
6	Grade II	Cs/PSL-FK506/ mPSL	ND	+/+/+	18	19	1	No
7	Grade II	Cs/PSL	ND	+/+/+	80	83	3	No
8	Grade II	PSL	11	+/+/+	53	109	56	AraA
9	No	FK506/PSL	11	+/+/+	25	25	0	No
10	Grade III	FK506	11	+/+/+	26	39	13	No
11	Chronic lung	FK506/PSL	11	+/+/+	142	149	7	No
12	No	FK506	11	+/+/+	34	34	0	No
14	Grade II	FK506/PSL	11	+/+/+	126	129	3	No
15	Grade II	Cs/PSL	11	+/+/+	50	64	14	No
16	NE	PSL	11	+/+/+	31	31	0	No

AdV = adenovirus; HC = hemorrhagic cystitis; CDV = cidofovir; IC = immunochromatography; NE = not evaluable; ND = not determined; GVHD = graft-versus-host disease; FK506 = tacrolimus; Cs = cyclosporine; PSL = prednisolone; AraA = vidaravin.

Table 3 Outcome of CDV treatment

Patient no.	*Improvement of HC*	*Onset of effect (days)*	*Eradication of ADV from the urine**	*Initial creat (mg/dl)*	*Max creat (mg/dl)*	*Final creat (mg/dl)*	*Renal toxicity (NCI-CTC)*	*Previous PFA treatment*	*Activation of herpesviruses during CDV treatment*
1	Effective	6	Effective	1.38	1.38	1.02	1→1	No	None
3	No	—	Effective	0.54	0.83	1.02	0→0	No	None
4	Effective	7	Effective	0.76	1.02	0.59	0→1	No	None
5	No	—	Effective	1.2	2.38	1.79	1→2	No	CMV antigenemia, HSV stomatitis
6	Effective	13	Effective	0.54	0.59	0.59	0→0	No	CMV antigenemia
7	Effective	12	Effective	0.89	0.97	0.59	0→0	No	None
8	No	—	No	1.2	5.3	5.3	1→3	Yes	None
9	Effective	9	Effective	0.56	1.21	0.83	0→1	No	None
10	Effective	9	Effective	0.35	0.41	0.41	0→0	No	None
11	No	—	No	1.3	2.8	2.8	1→2	No	None
12	Effective	14	Effective	1	1.38	1.03	0→1	Yes	CMV antigenemia, HSV stomatitis
14	Effective	14	Effective	0.8	1.2	1	0→1	No	None
15	Effective	10	Effective	1.4	1.4	0.9	1→1	No	None
16	Effective	8	Effective	1.05	1.34	0.67	1→1	No	CMV antigenemia

*The eradication of AdV was defined by negative culture for AdV 1 week after the last dose of CDV.
Initial creat = serum creatine when starting CDV treatment; Max creat = maximal serum creatine during CDV treatment; Final creat = serum creatine upon completing CDV treatment; CDV = cidofovir; PFA = foscarnet; HC = hemorrhagic cystitis; AdV = adenovirus; CMV = cytomegalovirus; HSV = herpes simplex virus.

while 1 g was given 1 and 8 h afterward. Intravenous hydration with normal saline also was given. Patients were followed up for 2 months after the completion of CDV treatment.

Results

Outcome of CDV therapy

CDV therapy was successful in clearing AdV from the urine in 12 of 14 patients (86%), as defined by negative culture for AdV 1 week after the last dose of CDV. Of 14 patients, 10 (71%) showed clinical improvement in HC (Table 3). Median time to improvement of HC grade after CDV therapy was 9.5 days (range, 6–14; Table 3 and Figure 1). Patients No. 3 and No. 5 had persistent symptoms of HC despite eradication of AdV in the urine.

Toxicity

Serum creatinine concentration for all patients, at the time of initiation and termination of CDV treatment, as well as the maximum serum creatinine concentration during CDV treatment, are shown in Table 3. Renal toxicity was graded according to the Common Toxcity Criteria of National Cancer Institute (NCI-CTC Version 2.0; April 30, 1999). Among 14 patients, seven (50%) had no renal toxicity.

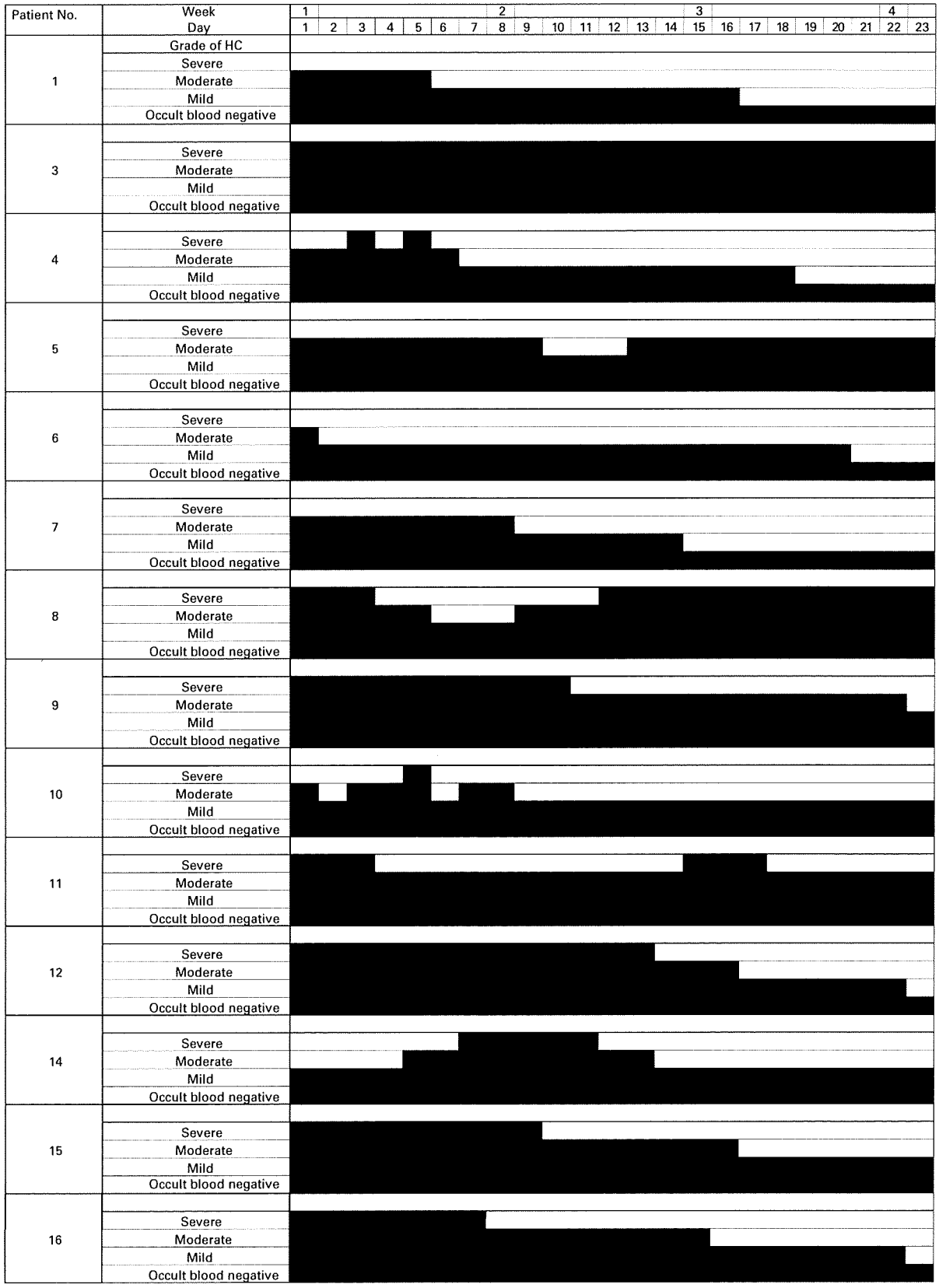

Figure 1 Clinical courses of 14 patients with adenoviral HC, who received cidofovir treatment.

Patient No. 8, who had been treated with foscarnet for CMV antigenemia, required hemodialysis and discontinuation of CDV treatment. Patient No. 11 had grade 2 renal toxicity and CDV treatment was terminated. In contrast, patient No. 5 had grade 2 renal toxicity, but could continue CDV treatment. Patient No. 10 developed veno-occlusive disease (VOD) during CDV treatment.

Virally associated findings during the treatment with CDV

Table 1 showed donor/recipient CMV and recipient HSV serostatus. As CDV has been reported to have significant anti-CMV and anti-HSV activity, concurrent use of acyclovir (ACV) or GCV was avoided to reduce renal toxicity. Patient No. 5 developed CMV antigenemia when CDV treatment was started, and CMV antigenemia persisted during CDV treatment. After completion of CDV treatment, he was treated with GCV, which abolished CMV antigenemia. Before CDV treatment, patient No. 6 was treated with GCV for CMV antigenemia that persisted throughout CDV therapy. Patient No. 12 developed CMV antigenemia during CDV treatment. After completion of CDV treatment, CMV antigenemia was abolished by treatment with foscarnet. Patient No. 16 developed CMV antigenemia during CDV treatment, and because of an increase in CMV antigenemia GCV was added. During CDV treatment, patients No. 5 and No. 12 developed HSV-1 stomatitis, which was treated successfully with ACV (Table 3).

Discussion

The present study reports the outcome in AdV HC treated with CDV. As expected, the main toxicity of CDV treatment was renal. Among 14 evaluable patients, two developed severe renal toxicity, resulting in discontinuation of CDV treatment. One of these patients who required hemodialysis had a history of foscarnet treatment. Previous treatment with foscarnet has been reported to exacerbate CDV renal toxicity,[23] which was proved for this patient. CDV renal toxicity complicating treatment of AdV HC is difficult to evaluate. Many other nephrotoxic agents, including cyclosporine, tacrolimus, and amphotericin B, are frequently administered to HSCT patients; furthermore, AdV infection itself can cause renal damage such as nephritis[16] and obstructive nephropathy.[24] In this study, six patients (Nos. 5, 9, 12, 14, 15, and 16) experienced increased level of serum creatinine concentrations, but continued CDV treatment, with improvement in terms of both AdV HC and renal function (Table 3). Thus, AdV HC itself may have contributed to the increase in serum creatinine during CDV treatment. Use of CDV before emergence of renal damage from AdV infection would be desirable. Patient No. 10 developed VOD, which has not been reported previously as a form of CDV toxicity. More information is necessary to determine whether or not VOD is among CDV toxicities.

Among 14 evaluable patients, 10 (71%) showed clinical improvement of AdV HC, which is similar to a success rate of 63% reported in patients with definite AdV disease reported by the European Group for Blood and Marrow Transplantation.[25] A long delay between AdV infection and treatment has been linked to a greater risk of treatment failure.[17] For rapid diagnosis, we used immunochromatography. At the onset of HC, all patients in the study were positive for AdV by this method. Positivity was confirmed later both by PCR result and by isolation of AdV from urine. Thus, immunochromatography appears reliable for rapid diagnosis of AdV HC. Since post-transplant AdV infection causes significant mortality[6,9–11] and HC causes considerable patient discomfort, CDV would appear to be beneficial treatment while maintaining an acceptable toxicity profile.

At a dose of 5 mg/kg/week, CDV has been reported to have significant anti-CMV and anti-HSV activity.[26] Indeed, CDV is considered a second-line treatment for GCV-refractory CMV disease.[27] Among our patients, two had persistent CMV antigenemia and two developed CMV antigenemia during treatment with CDV. In addition, two patients developed HSV-1 stomatitis. Thus, CDV at a dose of 1 mg/kg/day three times weekly may be insufficient to prevent or treat CMV or HSV disease. Alternatively, patients who develop AdV HC might be immune compromised to the extent that for them CDV treatment may not be effective against CMV or HSV. Vigilance against infection by and/or additional prophylaxis agents for herpesviruses, therefore, is important during CDV treatment with 1 mg/kg three times weekly.

In conclusion, CDV at a dose of 1 mg/kg/day, three times weekly could be administered with acceptable toxicity for effective treatment of AdV HC. Prospective randomized trials are necessary to further study the use of CDV for AdV HC.

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CASE REPORT

HLA-haploidentical nonmyeloablative stem cell transplantation: induction to tolerance without passing through mixed chimaerism

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Summary There are few reports of unmanipulated HLA-haploidentical nonmyeloablative stem cell transplantation (NST) using only pharmacological acute graft-*vs.*-host disease (GVHD) prophylaxis. We present here a successful case of unmanipulated HLA-haploidentical NST for mediastinal large B cell lymphoma that was resistant to autologous peripheral blood stem cell transplantation (PBSCT). The conditioning regimen consisted of fludarabine, busulfan and rabbit anti-T-lymphocyte globulin (ATG) in addition to rituximab. GVHD prophylaxis was performed using tacrolimus and methylprednisolone 1 mg/kg. The patient had rapid engraftment, with 100% donor chimaerism in the lineages of both T cells and granulocytes on day +12, but developed no GVHD clinically. The patient is still in complete remission past day +1020, with no sign of chronic GVHD without receiving immunosuppressive agents. HLA-haploidentical NST may be performed without utilizing mixed chimaerism.

Keywords HLA-haploidentical nonmyeloablative stem cell transplantation, graft-*vs.*-host disease, graft-*vs.*-lymphoma effect, non-Hodgkin's lymphoma

Introduction

There are only a few reports describing nonmyeloablative stem cell transplantation (NST) from human leucocyte antigen (HLA)-haploidentical donors (Sykes *et al.*, 1999; O'Donnell *et al.*, 2002). We recently showed that the combination of fludarabine, busulfan and anti-T-lymphocyte globulin (ATG), a reduced-intensity regimen (Slavin *et al.*, 1998), enabled engraftment of HLA-haploidentical related transplants (one antigen-mismatch in the graft-*vs.*-host (GVH) direction) (Tamaki *et al.*, 2003). However, in that study, acute graft-*vs.*-host disease (GVHD) could not be sufficiently controlled using GVHD prophylaxis with cyclosporine or tacrolimus (FK506) with or without mycophenolate mofetil. On the contrary, a protocol for HLA-haploidentical NST from 2 to 3 antigen-mismatched donors in the GVH direction without T-cell depletion using more intensified GVHD prophylaxis [FK506 + methylprednisolone (mPSL)] is now being tested in an ongoing study. Among the patients in that ongoing study, we recently encountered a patient with mediastinal large B-cell lymphoma (MLBCL) that was resistant to

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autologous peripheral blood stem cell transplantation (PBSCT), who was successfully treated by HLA-haploidentical NST, and is still in complete remission past day +1020 without receiving immunosuppressive agents.

Case report

A 26-year-old female developed bulky mediastinal and lung masses in May 2000 and was diagnosed with MLBCL. After she received three courses of CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), left upper lobectomy was performed for the residual lung mass. Four months later, lymphoma relapsed at the original mediastinal lesion. The patient received two additional courses of CHOP and four courses of Pro-MACE-CytaBOM (cyclophosphamide, doxorubicin, etoposide, cytaribine, bleomycin, vincristine, methotrexate with leucovorin, prednisone) with some effect, but the lymphoma soon began to grow again. Therefore, autologous PBSCT was performed using a preconditioning regimen consisting of cyclophosphamide, etoposide and ranimustine, but residual tumours remained in the left lung field and in the anterior mediastinum.

As there was no time to locate an unrelated donor, we decided to perform stem cell transplantation (SCT) from her sister with HLA-2-antigen mismatching in both the GVH and host-*vs.*-graft (HVG) directions (patient A24 B61 DRB1 0901/A24 B48 DRB1 0405, donor A2 B54 DRB1 0405/A24 B48 DRB1 0405). Institutional review board approval was obtained for the treatment protocol, and written informed consent was obtained from the patient and her family members, including the donor.

As the patient had already been heavily treated with chemotherapeutic drugs in addition to autologous PBSCT, a reduced intensity regimen was used. Clinical course of the patient was given in Figure 1. The preparative regimen consisted of fludarabine 30 mg/m^2 × 6 on days −10 to −5, oral busulfan 4 mg/kg on days −6 and −5, and rabbit ATG (Fresenius, Gräfelfing, Germany) 1.5 mg/kg × 4 on days −4 to −1. Rituximab 375 mg/m^2 was given on days −12, −5, +2 and +37. GVHD prophylaxis was performed by treatment with FK506 (the target blood concentration was 10–15 ng/ml) from day −3 and with mPSL 1 mg/kg starting from day 11. The patient underwent transplantation of peripheral blood stem cells containing 22 × 10^6 CD34$^+$ cells/kg without any manipulation. Granulocyte colony-stimulating factor 10 μg/kg/day was given from day +8. Haematopoietic reconstitution was rapid, with absolute neutrophil count >0.5 × 10^9/l on day +11 and platelet count >50 × 10^9/l on day +12. On day +12, complete donor chimaerism was confirmed in the cell lineages of both CD3$^+$ cells and granulocytes in the peripheral blood. The patient had no acute or chronic GVHD. The patient had no other post-transplant complications other than haemorrhagic cystitis, which improved after conventional treatments. Chest computed tomography scans performed on day +118 revealed that residual tumours present before NST had disappeared. On day +1020, the patient was still in complete remission with no sign of chronic GVHD without receiving immunosuppressive agents.

Discussion

To date, only a few studies of unmanipulated NST from HLA-haploidentical (mismatch of >1 antigen) donors have been reported. Sykes and colleagues performed transplants in four patients with refractory non-Hodgkin's lymphoma

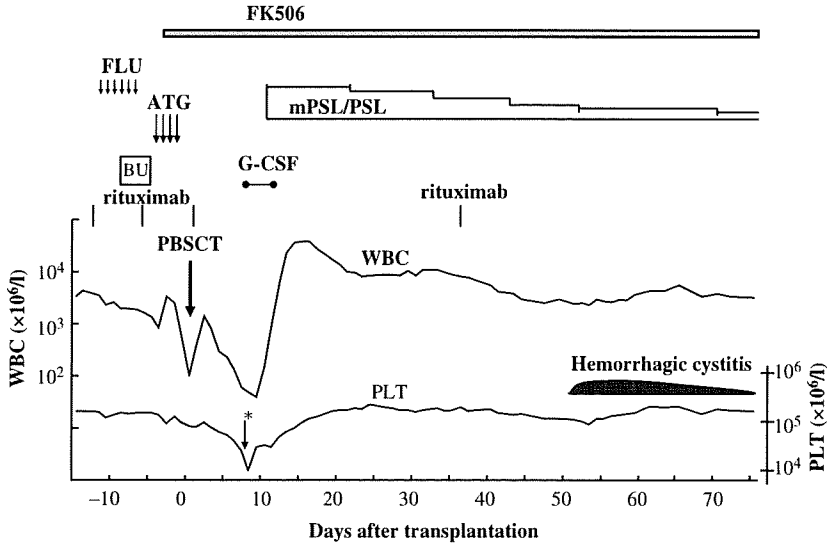

Figure 1. Clinical course of the patient. The bold and thin lines represent the white blood cell (WBC) and platelet (PLT) counts respectively. *The arrow denotes platelet transfusion, which was needed only once. mPSL, methylprednisolone; PSL, prednisone; FK506, tacrolimus; FLU, fludarabine; BU, busulfan; ATG, anti-T-lymphocyte globulin; PBSCT, peripheral blood stem cell transplantation.

using a reduced conditioning regimen (Sykes *et al.*, 1999). They focused on mixed lymphohaemopoietic chimaerism that was achieved across HLA barriers by a combination of CPA, thymic irradiation and ATG, a conditioning that they expected to specifically target host immune resistance. All patients achieved mixed chimaerism, but all except one, who was alive in complete remission on day +460, died of transplant-related toxicity or progressive disease within a short period of time after transplantation. O'Donnell and colleagues reported 10 patients who underwent HLA 2- or 3-antigen-mismatched BMT with a nonmyeloablative regimen including fludarabine, total body irradiation (2 Gy) and post-transplantation CPA (O'Donnell *et al.*, 2002). Their protocol was characterized by the use of high-dose CPA early after BMT, which was expected to attenuate both the GVH and HVG reactions. Mixed lymphohaemopoietic chimaerism was achieved after transplantation in their study; half of the patients, however, had graft rejection.

The investigators in those studies aimed to utilize mixed chimaerism for achieving bi-directional (GVH and HVG) tolerance. In contrast, the case in the present report suggests that both of these barriers can be overcome by the use of a conventional combination of preparative regimen and pharmacological GVHD prophylaxis even if the patient rapidly achieves 100% donor chimaerism. This rapid engraftment may have been caused by the use of PBSCs in our case (*vs.* the use of BM in studies cited above), in addition to sufficient immunosuppression brought about by the combination of fludarabine, busulfan and ATG.

At present, we do not know which is better, the achievement of rapid donor engraftment or mixed chimaerism in HLA-haploidentical NST settings. However, this successful case suggests that the GVH and HVG barriers in HLA-haploidentical transplantations can be overcome using a reduced preconditioning regimen (fludarabine + busulfan + ATG) and appropriate pharmacological GVHD prophylaxis. However, the feasibility of this transplant strategy must be investigated in a large-scale study.

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HEMATOLOGY

Reduced-Intensity Conditioning Followed by Unrelated Umbilical Cord Blood Transplantation for Advanced Hematologic Malignancies: Rapid Engraftment in Bone Marrow

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Abstract

Reduced-intensity (RI) conditioning followed by cord blood transplantation (CBT) is a new treatment modality, but failure to engraft is a major concern. We describe 12 patients with advanced hematologic malignancies who underwent RI conditioning and CBT with a conditioning regimen consisting of 200 mg/m^2 fludarabine (Flu), 50 mg/kg cyclophosphamide (CY), and 3 Gy total body irradiation (TBI). Cyclosporin A and/or methotrexate were used for graft-versus-host disease prophylaxis. Cord blood grafts were not mismatched for more than 2 serologically defined HLA alleles but were later found by high-resolution DNA typing to be mismatched for 2 to 4 alleles in most cases. Short tandem repeat analysis of bone marrow cells at day 14 showed complete donor chimerism in 6 of the patients and mixed chimerism in 5, indicating rapid engraftment in the bone marrow, whereas the remaining patient experienced graft rejection. Neutrophil recovery was achieved at a median of day 17 (range, days 11-24) in 10 of the 11 patients with marrow chimerism at day 14. Of these 10 patients, however, transplantation-related mortality within 100 days occurred in 4 patients who showed failed platelet recovery and a lack of durable engraftment. Overall survival and disease-free survival rates were 41.7% and 33.3%, respectively. These results show that CB mismatched at 2 to 4 HLA alleles and transplanted with the Flu/CY/3 Gy TBI regimen is able to engraft in the bone marrow as early as day 14.
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Key words: Hematologic malignancy; Cord blood transplantation; Adult; Reduced-intensity conditioning

1. Introduction

Allogeneic hematopoietic stem cell transplantation (SCT) is a potentially curative treatment modality for patients with hematologic malignancies [1]. Much of the therapeutic potential of SCT relates to the graft-versus-tumor (GVT) effect, in which donor T-cells mediate the eradication of the host malignancy [2]. Conventional myeloablative conditioning regimens, including high-dose chemotherapy and lethal total body irradiation (TBI), usually exert antitumor effects as well, and the intensive immunosuppression of these regimens allows the establishment of complete donor chimerism. However, these regimens are often accompanied by significant side effects, termed *regimen-related toxicity* (RRT) [3]. To minimize RRT, investigators have explored reduced-intensity (RI) preparative regimens for older patients or for patients with a poor performance status, organ dysfunction, or extensive prior therapy [4-6]. Grafting with RI conditioning regimens still facilitates a GVT effect.

Umbilical cord blood (CB) is increasingly being used as a source of stem cells for SCT as an alternative to bone marrow or peripheral blood stem cells harvested from human leukocyte antigen (HLA)-matched siblings or unrelated donors [7-8]. Advantages of CB as donor cells include the immediate availability of cryopreserved cells, less strict requirements for HLA matching between donor and recipient, and a low risk of inducing severe graft-versus-host dis-

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ease (GVHD). However, a major disadvantage of CB transplantation (CBT) is the availability of only a limited number of cells from a single CB donor, often resulting in graft failure and poor survival prospects for adult patients.

The combined use of RI regimens and CBT (RI-CBT) represents a new treatment modality for hematologic malignancies in patients without suitable marrow donors or patients otherwise not eligible for myeloablative SCT [9]. However, there are few reports thus far on RI-CBT for adult patients [10-12], and the optimal RI preparative regimen for CBT has not been firmly established. A major problem to be addressed in RI-CBT is graft failure. Barker et al [10] reported that a regimen of fludarabine (Flu), cyclophosphamide (CY), and 2 Gy of total body irradiation (TBI) (Flu/CY/2 Gy TBI regimen) enabled rapid and efficient engraftment of donor cells in RI-CBT. Here we report the results of the use of RI-CBT with a Flu/CY/3 Gy TBI conditioning regimen to treat 12 patients with advanced hematologic malignancies.

2. Patients and Methods

2.1. Patient Eligibility

Patients enrolled in this study had organ dysfunction, extensive prior chemotherapy, or a poor performance status. These patients did not have a related donor matched at 6 of 6 or 5 of 6 HLA alleles or an unrelated donor matched at 6 of 6 HLA alleles, or they needed urgent SCT within 3 months. All patients provided written informed consent. This pilot study was approved by the Institutional Review Board of Hyogo College of Medicine.

2.2. CB Grafts

CB was sought through the Japan Cord Blood Bank Network, which consists of 11 local cord blood banks in Japan [13]. A CB graft was selected on the basis of serologic matching for 4 to 6 alleles at 3 HLA loci (class I HLA-A and HLA-B, and class II HLA-DR), as determined by a standard complement-dependent microlymphocytotoxicity technique [14]. Selected CB grafts had a cryopreserved cell dose of at least 2×10^7 nucleated cells/kg recipient body weight. When multiple suitable CB units were available, we selected the unit that displayed the highest nucleated cell dose from the units that had no more than 2 HLA mismatches. Prior to shipping from the local cord blood bank, confirmatory high-resolution DNA typing of class I HLA-A and HLA-B alleles and class II DRB1 alleles was performed by means of polymerase chain reaction (PCR) analysis with sequence-specific primers [15], PCR analysis with sequence-specific oligonucleotide probes [16], or PCR-based single-strand conformation polymorphism analysis [17]. All of the CB aliquots used were single units and not depleted of T-lymphocytes.

2.3. Preparative Regimen

The preconditioning regimen (the Flu/CY/3 Gy TBI regimen) consisted of 50 mg/kg CY on day –6, 40 mg/m^2 Flu daily for 5 days from days –6 to –2, and 3 Gy TBI (in 2 fractions on day –1). GVHD prophylaxis consisted of a continuous infusion of 3 mg/kg cyclosporin A (CsA) from day –3 until the patients tolerated oral administration. After neutrophil engraftment and in the absence of acute GVHD, CsA was tapered 10% per week starting at approximately day 35. This early tapering of CsA treatment was intended to induce a graft-versus-leukemia effect through attenuated GVHD prophylaxis [18]. Although short-term methotrexate treatment was administered at 10 mg/m^2 (day 1) and 7 mg/m^2 (days 3 and 6) in the first 3 patients, it was omitted in subsequent patients in this context. Granulocyte colony-stimulating factor was administered to all patients at 5 μg/kg per day from day 1 until neutrophil recovery.

2.4. Donor Chimerism Analysis

Donor chimerism was analyzed in marrow and/or blood samples on days 14, 21, 35, and 100, or as clinically dictated. Chimerism was determined by quantitative PCR analysis of informative short tandem repeat (STR) regions in the recipients and donors (STR-PCR) [19]. DNA was extracted from bone marrow or blood cells by means of a SepaGene DNA isolation kit (Sanko Junyaku Co, Tokyo, Japan) and amplified with fluorescent PCR primers (AmpFLSTR Profiler PCR amplification kit; Applied Biosystems, San Jose, CA, USA). The fluorescent PCR products were separated by capillary electrophoresis using a 310 Genetic Analyzer (Applied Biosystems). GeneScan and GeneMapper software packages (Applied Biosystems) were used to calculate the percentages of donor and recipient DNA. Complete (donor) chimerism was defined as the detection of 100% donor DNA in a sample.

2.5. Engraftment

Engraftment was defined when white blood cell counts $>1.0 \times 10^9$/L or absolute neutrophil counts $>0.5 \times 10^9$/L were obtained for 3 consecutive days after transplantation and the attainment of these count thresholds was accompanied by the detection of donor chimerism. Bone marrow engraftment was defined as donor chimerism in the bone marrow detected by STR-PCR analysis, even if the peripheral blood cell count did not reach the engraftment level described above. Primary graft failure (rejection) was defined as peripheral and marrow hypoplasia occurring after transplantation without the detection of donor markers by cytogenetic and/or molecular techniques and was estimated at approximately day 30.

2.6. Graft-versus-Host Disease

Acute GVHD was clinically diagnosed by means of the criteria described by Glucksberg et al [20]. Patients who survived >100 days were evaluated for chronic GVHD.

2.7. RRT and Transplantation-Related Mortality

RRT was defined as any nonhematologic organ dysfunction from day 0 to day 28 [3]. Transplantation-related mortality (TRM) was defined as death without progression of the primary disease.

Table 1.
Characteristics of Patients*

Patient No.	Age, y/Sex	Weight, kg	Disease	Status at Transplantation	HLA Disparity†		GVHD Prophylaxis	NC, $\times 10^7$/kg	CD34, $\times 10^6$/kg
					Serology	DNA Typing			
1	40/F	57	NHL-REL	Refractory‡	5/6 (B)	4/6 (A, B)	CsA/MTX	2.26	0.59
2	49/F	54	AML-CR	CR§	4/6 (A, A)	3/6 (A, A, DR)	CsA/MTX	2.26	0.74
3	35/M	58	AML-CR	CR§	4/6 (B, DR)	4/6 (B, DR)	CsA/MTX	2.29	0.80
4	23/F	53	AML-REL2	Refractory§	4/6 (B, DR)	4/6 (B, DR)	CsA	2.35	0.87
5	34/M	67	NHL-REL2	Refractory‡	4/6 (B, DR)	4/6 (B, DR)	CsA	2.52	0.87
6	55/F	48	ATL-REL	Refractory	4/6 (A, DR)	3/6 (A, DR, DR)	CsA	2.55	0.91
7	55/F	62	MDS-REL2	Refractory	6/6	6/6	CsA	2.60	0.91
8	19/M	61	ALL-REL	Refractory§	4/6 (B, DR)	4/6 (B, DR)	CsA	2.63	0.92
9	56/M	57	MDS-REL	Refractory	4/6 (B, DR)	3/6 (A, B, DR)	CsA	2.64	0.97
10	63/M	72	MDS-REL	Refractory	5/6 (B)	4/6 (B, DR)	CsA	2.71	0.98
11	55/M	63	ATL-REL1	Refractory	5/6 (DR)	3/6 (A, B, DR)	CsA	2.85	1.27
12	62/M	61	MDS-REL	Refractory	4/6 (B, DR)	2/6 (A, B, DR, DR)	CsA	3.33	2.04

*GVHD indicates graft-versus-host disease; NC, nucleated cells; CD34, $CD34^+$ cells; NHL, non-Hodgkin's lymphoma; REL, relapse; CsA, cyclosporin A; MTX, methotrexate; AML, acute myelogenous leukemia; CR, complete remission; ATL, adult T-cell leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia.

†HLA disparities between donor and recipient are shown in parentheses.

‡Patient who had undergone previous radiation therapy.

§Patient who had undergone previous allogeneic stem cell transplantation.

3. Results

3.1. Patient Characteristics

Twelve patients underwent RI-CBT between August 2003 and July 2004 at our institution. The patients' characteristics are presented in Table 1. The diagnoses included acute myelogenous leukemia (n = 3), acute lymphoblastic leukemia (n = 1), non-Hodgkin's lymphoma (n = 2), myelodysplastic syndrome (n = 4), and adult T-cell leukemia (n = 2). Ten (83%) of the 12 patients had refractory or relapsed disease, indicating that they had a poor prognosis. The other 2 patients had relapsed after their first allogeneic SCT and then achieved advanced complete remission. The median age of the patients was 49 years (range, 19-63 years), and the median weight was 58 kg (range, 48-72 kg).

3.2. Graft Characteristics

The median number of nucleated cells infused was 2.55×10^7/kg body weight (range, $2.26\text{-}3.33 \times 10^7$/kg), and the median number of infused $CD34^+$ cells was 0.91×10^5/kg body weight (range, $0.59\text{-}2.04 \times 10^5$/kg) (Table 1). Assessment of HLA disparity using both standard serologic methods and high-resolution DNA typing is shown in Table 1. We originally selected CB mismatched for no more than 2 serologically defined HLA alleles. However, DNA analysis revealed 1 or 2 additional HLA mismatches in 7 of the 12 patients. Therefore, 92% of the CB grafts in fact were HLA mismatched for 2 to 4 alleles.

3.3. Engraftment and Chimerism

The clinical outcomes for all patients are summarized in Table 2. We used an RI regimen that was slightly modified from the protocol reported by Barker et al [10] by increasing the TBI dose from 2 to 3 Gy. This modification was made because the patients in our previous RI-CBT pilot study received 2 Gy TBI with Flu and they experienced graft rejection. All patients in the present study experienced pancytopenia after RI-CBT. Although the peripheral blood neutrophil count was less than 500/μL on day 14 in most patients, STR analysis of bone marrow cells on day 14 revealed complete (100%) donor chimerism in 6 of the 12 patients, and 20% to 90% donor chimerism in another 5 patients (Table 2). These results showed unexpectedly early engraftment of donor cells in the bone marrow after the Flu/CY/3 Gy TBI regimen of RI-CBT. The remaining patient (no. 2) experienced graft rejection with the recovery of autologous hematopoiesis. With the exception of 1 patient who was HLA matched, the actual HLA disparity in the other 11 patients was found by genetic typing to be 2 to 4 alleles. Eleven patients showed chimerism in the bone marrow at day 14, and 10 of these patients achieved neutrophil recovery (>500/μL) at a median of 17 days (range, 11-24 days). Platelet recovery was achieved at a median of 32 days (range, 26-44 days) in 5 of the 10 patients who showed neutrophil recovery (Table 2).

3.4. Event-Free and Overall Survival

With a median follow-up of 13 months, the median overall survival time of the entire cohort was 493 days (range, 426-565 days). Overall survival and disease-free survival rates of these 12 patients were 41.7% and 33.3%, respectively (Figure 1).

3.5. Graft-versus-Host Disease

The cumulative incidence of acute GVHD of grade II to IV in these patients conditioned with the Flu/CY/3 Gy TBI regimen was 62.5%. Thirty-three percent of the patients developed chronic GVHD (Table 2).

Table 2.
Outcome of Patients after Reduced-Intensity Conditioning and Cord Blood Transplantation*

Patient No.	Donor Engraftment in Bone Marrow, %			Time to Neutrophils >0.5 × 10^9/L, d	Time to Platelets >20 × 10^9/L, d	Acute GVHD	Chronic GVHD	Current Status	Cause of Death
	Day 14	Day 35	Day 100						
1	17	100	100	24	44	I	No	Dead, d 412	Disease progression
2	0	0	0	Never	Never	NE	NE	Alive, d 565+	
3	72	100	100	20	32	I	Limited	Alive, d 564+	
4	53	100	100	11	37	II	No	Alive, d 493+	
5	100	NE	NE	20	Never	NE	NE	Dead, d 23	Encephalopathy
6	100	100	NE	23	Never	III	NE	Dead, d 40	TMA
7	84	100	100	14	26	I	No	Alive, d 472+	
8	100	100	NE	16	Never	III	NE	Dead, d 71	TMA
9	100	100	NE	17	Never	II	No	Dead, d 117	GI bleeding
10	100	100	100	13	27	II	Limited	Alive, d 426+	
11	100	100	NE	17	Never	NE	NE	Dead, d 44	TMA
12	95	100	NE	Never	Never	NE	NE	Dead, d 37	TMA

*GVHD indicates graft-versus-host disease; Never, neutrophil count never becomes >0.5 × 10^9/L, or platelet count never becomes >20 × 10^9/L; NE, not evaluable; TMA, thrombotic microangiopathy; GI, gastrointestinal.

3.6. RRT and TRM

No patient developed obvious RRT as defined by the criteria reported by Bearman et al [3]. However, the TRM rate before day 100 in patients conditioned with this Flu/CY/3 Gy TBI regimen was 41.7% (5 of 12 patients). Causes of death were central nervous system complications (encephalopathy) (n = 1) [21] and thrombotic microangiopathy (n = 4).

4. Discussion

An essential mechanism of the efficacy of SCT is related to the GVT effect, in which donor T-cells eradicate tumor cells [1,2]. RI preparative regimens for SCT aim to reduce RRT and enhance GVT effects in the treatment of hematologic malignancies [4,5]. The use of CB has markedly expanded the application of SCT to include patients without HLA-matched donors [7,8]. In particular, SCT using an RI-CBT protocol should be applicable to a number of patients not eligible for conventional HLA-matched myeloablative SCT [9]. However, the optimal regimen for RI-CBT has not yet been firmly established.

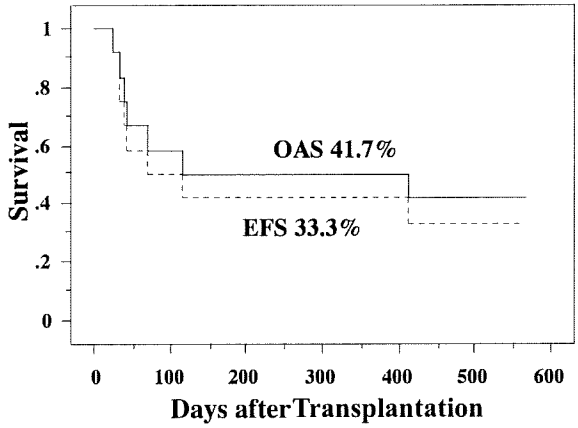

Figure 1. Kaplan-Meier estimates of overall survival (OAS) and event-free survival (EFS).

A major concern regarding CBT with RI conditioning for adult patients is graft failure. Barker et al [10] reported that when 43 adult patients with high-risk or advanced hematologic malignancies were given single- or double-unit CB infusions after conditioning with 2 different Flu-based regimens, engraftment was established in 30 patients with a low incidence of acute GVHD, despite the use of grafts mismatched at 1 or 2 HLA alleles, and resulted in 1-year disease-free survival rates of 21% to 41%. Using the slightly modified Flu/CY/3 Gy TBI regimen, we obtained a comparable survival rate (Figure 1), even though we used only a single unit of CB.

In conventional CBT, no more than 2 HLA mismatches at serologically defined class I HLA-A and HLA-B alleles and DNA-typed class II HLA-DRB1 alleles are considered the criterion for CB graft selection [22-24]. In RI-CBT, it is not clear how many HLA mismatches are acceptable, but most clinicians use CB mismatched at not more than 2 HLA alleles [10-12]. In our CB selection, all of the HLA disparity was at the level of 0 to 2 mismatches according to serologic HLA class I and class II typing. However, high-resolution DNA typing of class I HLA-A and HLA-B and class II DRB1 revealed that the HLA disparity was in fact 2 to 4 mismatches in most patients (Table 1). Thus, genetic HLA disparities were higher than those detected by serologic typing. It is clear that in unrelated CBT, high-resolution DNA typing reveals greater genetic disparity. The effect of this greater HLA genetic disparity on transplantation outcome should be analyzed in a future study.

Engraftment of donor cells is usually defined by neutrophil recovery in the peripheral blood. Early evaluation in the bone marrow before neutrophil recovery in the peripheral blood has not been described. In the present study, an analysis of bone marrow chimerism at day 14 showed unexpectedly early engraftment that had been previously unrecognized in RI-CBT. Early marrow engraftment was paralleled by neutrophil recovery in most patients, suggesting that day 14 bone marrow chimerism does predict donor engraft-

ment. The one exception in our study was a patient (no. 12) with severe infection in whom neutrophil recovery was aborted after early marrow engraftment (Table 1). Our results suggest that CB with 2 to 4 HLA mismatches is able to engraft in bone marrow as early as day 14 following preconditioning with the Flu/CY/3 Gy TBI regimen.

Overall survival and disease-free survival rates were 41.7% and 33.3%, respectively, in our patients (Figure 1). As is shown in Table 1, our patients were in markedly advanced stages of their hematologic malignancies. Ten of these patients had refractory disease, and 2 were in advanced complete remission after having relapsed following previous allogeneic SCT. Such patients have an extremely poor prognosis with conventional salvage therapy. Despite the low-intensity preparative regimen, the therapeutic responses obtained in the present study suggest that RI-CBT exerts antitumor activity through GVT effects. Overall survival will likely improve once the optimal conditions for RI-CBT in terms of types of hematologic malignancy, disease stage, and conditioning regimen are fully established.

The incidence of acute GVHD of grade II to IV in our patients was 62.5%, which is higher than the 44% incidence reported by Barker et al [10]. This high GVHD rate may be related to our early tapering of GVHD prophylaxis. Because most of our patients had refractory hematologic disease, we intended to eradicate the malignancy through the GVT effect by an early induction of GVHD [18]. The appearance of GVHD due to early tapering of GVHD prophylaxis seemed to be related to the occurrence of thrombotic microangiopathy (TMA). In our patients, the TRM rate within 100 days was 41% (Table 2). This rate is identical to that reported recently for RI-CBT in the treatment of malignant lymphoma [25]. The major causes of TRM in our patients were TMA and encephalopathy (Table 2). Kishi et al showed that central nervous system complications such as encephalopathy are fatal complications after RI-CBT [21]. We think that early tapering of GVHD prophylaxis induces acute GVHD and that this induction may be related to the subsequent appearance of TMA or encephalopathy. Furthermore, infection early after RI-CBT also seemed to be related the appearance of TMA. Additional strategies to enhance the GVT effect and reduce GVHD and TRM should therefore be developed.

Our study indicated that RI-CBT using Flu/CY/3 Gy TBI preconditioning allows early bone marrow engraftment and, despite the fact that our patient cohort was small, demonstrated the feasibility of RI-CBT for adult patients with hematologic malignancy. Our RI-CBT protocol is currently associated with a high TRM rate; therefore, further studies are needed to optimize this therapy to minimize the adverse effects and maximize GVT effects.

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Both perforin and Fas ligand are required for the regulation of alloreactive CD8$^+$ T cells during acute graft-versus-host disease

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Fas ligand (FasL) and perforin pathways not only are the major mechanisms of T cell–mediated cytotoxicity but also are involved in homeostatic regulation of these T cells. In the present study, we tested whether CD8$^+$ donor T cells that are deficient in both perforin and FasL (cytotoxic double deficient [cdd]) could induce graft-versus-host disease (GVHD) in a major histocompatibility complex class I–mismatched lethally irradiated murine model. Interestingly, recipients of cdd CD8$^+$ T cells demonstrated significantly greater serum levels of interferon gamma and tumor necrosis factor alpha and histopathologic damage from GVHD than wild-type (wt) T cells on day 30 after allogeneic bone marrow transplantation ($P < .05$). Wt and either perforin-deficient or FasL-deficient CD8$^+$ T cells expanded early after transplantation followed by a contraction phase in which the majority of expanded CD8$^+$ T cells were eliminated. In contrast, cdd CD8$^+$ T cells exhibited prolonged expansion and reduced apoptosis to alloantigen stimulation in vivo and in vitro. Together these results suggest that donor cdd CD8$^+$ T cells expand continuously and cause lethal GVHD, and that both perforin and FasL are required for the contraction of alloreactive CD8$^+$ T cells. (Blood. 2005;105: 2023-2027)

Introduction

Graft-versus-host disease (GVHD) is the major complication of allogeneic bone marrow transplantation (BMT). GVHD occurs when donor T cells recognize major histocompatibility complex (MHC) and their associated peptides on host-derived antigen-presenting cells (APCs).[1-3] The target organs of GVHD are the skin, gut, liver, and lymphohematopoietic compartments. We recently demonstrated that alloantigen expression on host target epithelium is not necessary to initiate GVHD in mouse models of BMT, and inflammatory cytokines play a central role in the CD4$^+$-mediated GVHD.[4] Interestingly, single cytotoxic-deficient (perforin or Fas ligand [FasL]) donor T cells can induce CD4$^+$-mediated GVHD,[5,6] and recently, studies have demonstrated that cytotoxic double-deficient (cdd) CD3$^+$ and CD4$^+$CD8$^-$ T cells can also effect GVHD in allogeneic minor histocompatibility antigen (mHA) or MHC-disparate BMT models.[5-7] CD8$^+$-dependent GVHD is reportedly induced by both cytokine- and cytolytic T lymphocyte (CTL)–mediated cytotoxicity as several studies using FasL- or perforin/granzyme-deficient mice suggested that CTL-mediated cytotoxicity plays a role in the CD8$^+$-mediated, MHC class I–mismatched GVHD model.[5,8,9]

Recent studies have demonstrated that FasL and perforin not only are the principal cytotoxic effector molecules, but also are involved in homeostatic regulation of CD8$^+$ T cells. The role of Fas/FasL in lymphocyte homeostasis was clearly established with the recognition that functional null mutations in these proteins were associated with exacerbated autoimmune disease.[10,11] Perforin-deficient mice have essentially normal immune homeostasis. However, considerable evidence indicates the existence of a perforin-dependent mechanism to regulate the magnitude of CD8$^+$ T cell expansion in models as diverse as GVHD, viral and bacterial infections, and dendritic cell (DC) immunization.[12-19] The present studies demonstrate that CD8$^+$ T cells lacking the major cytotoxic pathways can induce GVHD across an MHC class I–only disparity. Moreover, we also found that both perforin and FasL contribute to the regulation of alloreactive CD8$^+$ T cell expansion and contraction during the acute period of GVHD in this model.

Materials and methods

Mice

Female C57BL/6 (B6, H-2^b), B6.C-H2^{bm1}/ByJ (bm1), C57BL/6-*Prf1*^{tm1Sdz}/J (pfp$^{-/-}$, H-2^b), and B6Smn.C3-*Tnfsf6*^{gld}/J (*gld*, H-2^b) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The bm1 mice possess a mutant class I allele that differs from B6 mice. C57BL/6 cytotoxic double-deficient (perforin and FasL) mice (cdd, H-2^b) were generated from the breeding of *pfp*$^{+/-}$*gld* pairings as described previously.[7] The offspring were then screened for homozygous perforin deficiency by polymerase chain reaction (PCR) as previously reported[20,21] to select for *pfp*$^{-/-}$*gld* (ie, cdd). The cdd mice (6 to 8 weeks old) were maintained in pathogen-free conditions in the Department of Microbiology and Immunology at the University of Miami School of Medicine. *gld* and cdd mice were used as donors before the age of 9 weeks after a flow cytometric analysis confirmed

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that there was no evidence of accumulation of $CD3^+CD4^-CD8^-B220^+$ T cells in their spleens. The age range of mice used as other bone marrow (BM) transplant donors and recipients was between 8 and 12 weeks.

BMT

Mice underwent transplantation according to a standard protocol described previously.[4] Briefly, mice were irradiated again with 13 Gy total body irradiation (TBI), split into 2 doses, and injected with 2×10^6 $CD8^+$ splenic T cells with 5×10^6 T-cell–depleted (TCD) BM cells from wild-type (wt) B6 donors after 13-Gy total body irradiation (TBI). $CD8^+$ T cells were negatively isolated by using CD4, DX5, MHC class II, and CD11b Micro Beads and the auto magnetic-activated cell sorter (MACS) following nylon wool purification of T cells from splenocytes.

Systemic and histopathologic analysis of GVHD

Survival after BMT was monitored daily and the degree of clinical GVHD was assessed weekly by a scoring system that sums changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) as described.[22] This score is a more sensitive index of GVHD severity than weight loss alone in multiple murine models.[22] Acute GVHD was also assessed by detailed histopathologic analysis of liver, intestine, and skin, as described.[23,24] The degree of cell infiltration in tissue was assessed by a scoring system incorporating 4 parameters: cell infiltration in portal triada, bill ducts/ductules, vascular, and hepatocellular area. Slides were coded without reference to mouse type or prior treatment status and examined systematically by a single pathologist (C.L.) using a semiquantitative scoring system.[23,24]

Mixed leukocyte reactions

Splenic $CD8^+$ T cells and dendritic cells (DCs) were isolated using CD8 and CD11c Micro Beads, respectively. The purity of the CD8 T cell and DC suspension was more than 90%. $CD8^+$ T cells were used as responders at 2×10^5/well against irradiated (20 Gy) DCs (1×10^4/well) for 2 to 4 days. During the final 12 hours of culture, cells were pulsed with 1 μCi (0.037 MBq) [^{3}H] thymidine (2 Ci/mmol [74.0 GBq]; Perkin Elmer, Billerica, MA) and proliferation was determined on a Top Count NTX (Packard Instrument, Meriden, CT).

Flow cytometric analysis

A flow cytometric analysis was performed using fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, or allophycocyanin-conjugated monoclonal antibodies (mAbs) to mouse CD45.1, CD3, CD4, CD8, B220, and CD11c (BD Pharmingen, San Diego, CA). Cells were preincubated with 2.4G2 mAbs to block Fcγ receptor, and were then incubated with the relevant mAbs for 30 minutes at 4°C. Finally, cells were washed twice with 0.2% bovine serum albumin in phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde in PBS and analyzed by EPICS Elite ESP cell sorter (Beckman-Coulter, Miami, FL). Irrelevant immunoglobulin $G_{2a/b}$ mAbs were used as a negative control. For analysis of donor cell apoptosis, spleens from recipient mice were harvested 10 days after transplantation and stained with PE-CD8, then washed with PBS, and then stained with FITC–conjugated annexin (R&D Systems, Minneapolis, MN) in the dark for 15 minutes at room temperature. Donor cell apoptosis was identified based on double staining for CD8 and annexin.

Enzyme-linked immunosorbent assay (ELISA)

ELISA for interferon-gamma (IFN γ) and tumor necrosis factor-alpha (TNF α) (BD Pharmingen) was performed as described.[25] Samples and standards were run in duplicate.

Statistical analysis

The Mann-Whitney *U* test was used for the statistical analysis of in vitro data, while the Wilcoxon rank test was used to analyze survival data. Linear regression and analysis of covariance were used to quantify the relationship between 2 variables. *P* less than .05 was considered statistically significant.

Results

Cytotoxic double-deficient $CD8^+$ T cells cause acute GVHD

Previous studies have shown that lack of either the perforin ($pfp^{-/-}$) or FasL *(gld)* in donor T cells significantly reduced GVHD following nonmyeloablative conditioning in a murine model.[26] We evaluated the role of each pathway in donor $CD8^+$ T cells during GVHD following myeloablative conditioning in a donor-recipient strain combination that differs at a single MHC class I antigen.[27] bm1 mice received 13 Gy total body irradiation (TBI) on day 0 and then received transplants of 5×10^6 TCD BM from wt B6 and 2×10^6 $CD8^+$ T cells from either wt, *gld,* or $pfp^{-/-}$ B6 donors; survival at day 30 after BMT was similar in all donor groups (8/10, 8/10, and 9/10, respectively). We analyzed histologic changes of GVHD at day 30 in the liver and intestine using a semiquantitative pathology index ("Materials and methods"). As shown in Figure 1, GVHD damage in target organs did not differ among donor groups, although histologic damage in all groups was relatively high due to the young age of recipients. Therefore, the deficiency of a single cytotoxic effector molecule in $CD8^+$ donor T cells did not reduce GVHD in this BMT model following myeloablative conditioning.

We next evaluated cytotoxic double-deficient (cdd) $CD8^+$ T cells in this GVHD model. The bm1 mice received transplants as before from wt or cdd B6 donors. Surprisingly, cdd $CD8^+$ T cells caused more rapid mortality than wt donor cells (Figure 2A). Histologic GVHD damage in the liver and intestine was also significantly greater on day 30 after BMT in recipients of cdd T cells (Figure 2B). We next evaluated inflammatory cytokines associated with acute GVHD. Compared with wt cells, cdd donor T cells caused dramatic increases in serum levels of IFN γ and TNF α on day 30 even though single cytotoxic deficient donor cells did not (Figure 2C).

Cdd T cells exhibit prolonged expansion to alloantigen stimulation in vivo and in vitro

Because recipients of cdd cells showed dramatic increase in serum levels of inflammatory cytokines and recipients of single deficient cells did not, we hypothesized that a mechanism in addition to CTL toxicity might be operative in the induction of GVHD. FasL and perforin not only are important effector molecules of CTL, but they also are involved in homeostatic regulation of $CD8^+$ T cells.[17-19] In this light, we hypothesized that cdd T cells proliferated to a greater extent than wt cells, causing increased cytokine production and more severe GVHD. We thus compared in vivo expansion of donor T cells in recipients of allogeneic $CD8^+$ T cells from wt, *gld,*

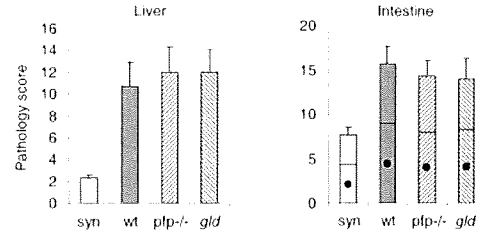

Figure 1. Effects of perforin- or FasL-deficient $CD8^+$ T cells on acute GVHD. Lethally irradiated bm1 mice received transplants as described in "Materials and methods" from wt B6 (wt), perforin-deficient B6 ($pfp^{-/-}$), or Fas ligand–deficient B6 *(gld)* donors. Syngeneic B6 BM transplant recipients (syn) served as no-GVHD controls. At 30 days after BMT, liver and intestine (bottom column [●], small intestine; upper column, large intestine) were harvested and scored semiquantitatively (n = 4 mice/group). Data represent mean ± SD.

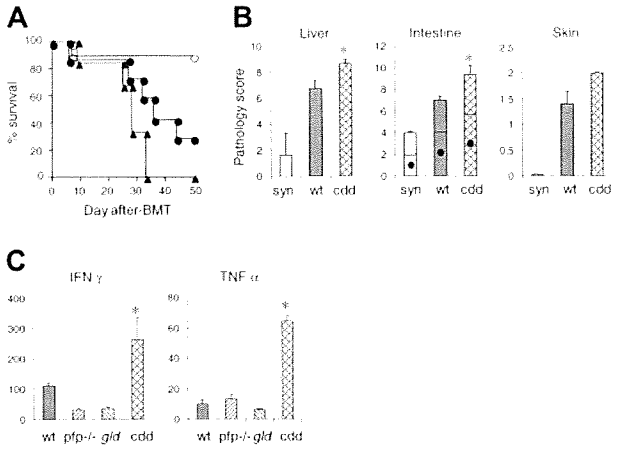

Figure 2. Cdd CD8⁺ T cells cause acute GVHD. The bm1 cells were transplanted from wt B6 (●, n = 11) or cdd B6 (▲, n = 6) donors as in Figure 1. B6 syngeneic BM transplant recipients (○, n = 9) served as no-GVHD controls. (A) Survival. (B) Histology. At 30 days after BMT, liver, intestine (bottom column [●], small intestine; upper column, large intestine), and skin were harvested and scored semiquantitatively (n = 4/group). B6 → B6 (syn; □), wt B6 → bm1 (wt; ▨), and cdd B6 → bm1 (cdd; ▩). (C) Serum cytokines. Mice received transplants of wt, pfp$^{-/-}$, *gld*, or cdd as in Figure 1 and were killed on day 30 (n = 3 mice/group). Serum was harvested and analyzed for TNF α and IFN γ by ELISA (pg/mL). Each graph represents 1 of 3 similar experiments. Data represent mean ± SD. wt versus cdd, *$P < .05$ by Mann-Whitney *U* test.

pfp$^{-/-}$, and cdd donors. T cells from wt donors expanded in the spleen until day 10 after BMT, followed by a rapid decline (contraction phase) (Figure 3A). *gld* T cells showed similar kinetics as wt T cells, whereas the pfp$^{-/-}$ T cells showed a significantly greater peak on day 10 but also contracted by day 30. By contrast, cdd CD8⁺ T cells expanded continuously up to day 30 after BMT, peaking at 100 times the number of wt T cells. Flow cytometric analysis of intracytoplasmic cytokines revealed that a greater proportion of cdd CD8⁺ T cells produced IFN γ and TNF α compared with wt T cells ($P < .05$, Figure 3B), consistent with the increased serum levels of these cytokines. We then analyzed whether GVHD damage correlated with the number of infiltrating mononuclear cells. We chose the liver for this evaluation because of the large dynamic range of cellular infiltration in this target organ, incorporating portal triads and bile ducts/ductules as well as vascular and hepatocellular areas. As shown in Figure 3C, the amount of hepatic tissue damage correlated closely with the degree of cellular infiltration ($P < .01$). Taken together, these data demonstrate that the absence of both cytotoxic pathways led to increased donor CD8⁺ T-cell expansion and greater cytokine production, causing greater GVHD target organ damage.

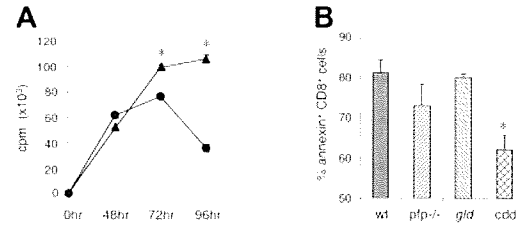

Figure 4. Deficiency of both perforin and FasL causes decreased apoptosis of donor CD8⁺ T cells after BMT. (A) Wild-type (●) and cdd (▲) CD8⁺ T cells at 2 × 10^5/well were cultured with irradiated bm1 DCs (1 × 10^4/well) for 2 to 4 days. During the final 12 hours of culture, cells were pulsed with [^{3}H] thymidine and assayed for proliferation. *$P < .05$ by Mann-Whitney *U* test. (B) Mice underwent transplantation as in Figure 1 and splenocytes were analyzed on day 10 after BMT by 2-color flow cytometry for expression of annexin and CD8⁺ cells. Gates were set for CD8⁺ cells, and the percentage of cells expressing annexin was determined. Data represent mean ± SD. wt versus cdd, *$P < .05$ by Mann-Whitney *U* test.

Deficiency of both perforin and FasL causes decreased apoptosis of donor CD8⁺ T cells after BMT

We next evaluated whether the increased expansion of cdd T cells was due to more rapid proliferation or to decreased activation-induced cell death (AICD). CD8⁺ T cells were isolated from wt and cdd mice and were cultured with DCs isolated from bm1 mice. Proliferation of both cell types was equivalent for the first 48 hours of culture. Proliferation of wt cells peaked at 72 hours after the initiation of culture and then declined; in contrast, proliferation of cdd CD8⁺ T cells continued to expand at least up to 96 hours (Figure 4A).

We next harvested spleens on day 10 after BMT and analyzed donor T cells by 2-color flow cytometry for annexin as a measure of apoptosis. As shown in Figure 4B, percentages of apoptosis of pfp$^{-/-}$ and *gld* CD8⁺ T cells were comparable with wt cells ($P = .27$ and .51, respectively). In contrast, cdd donor CD8⁺ T cells showed significantly less apoptosis ($P < .05$), demonstrating that impaired AICD in cdd T cells results in their greater expansion.

Discussion

Little is known regarding the regulation of the contraction phase of CD8⁺ T cells after alloantigen stimulation. The present study demonstrates that both perforin and FasL are required for the normal AICD-mediated contraction of activated CD8⁺ T cells during GVHD following myeloablative conditioning. Absence of either of these pathways alone did not affect AICD and did not reduce GVHD. Our findings are consistent with those of Spaner et al[12] and others who reported that pfp$^{-/-}$ CD8⁺ T cells proliferate

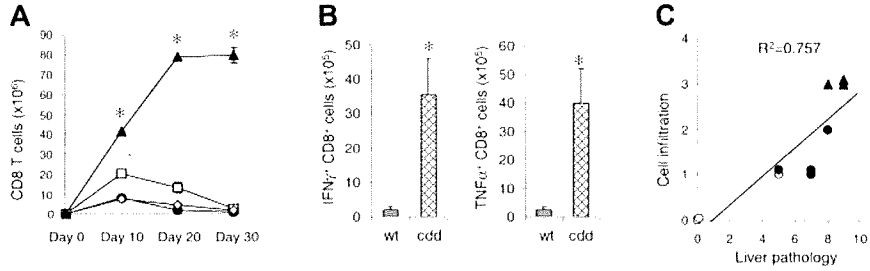

Figure 3. Deficiency of both perforin and FasL in donor CD8⁺ T cells cause prolonged expansion after BMT. (A) Mice underwent transplantation as in Figure 1. Splenocytes were harvested 10, 20, and 30 days (n = 3 mice/group) after BMT and analyzed by fluorescence-activated cell sorter (FACS). wt B6 → bm1 (●), pfp$^{-/-}$ → bm1 (□), *gld* → bm1 (◇), and cdd → bm1 (▲). wt B6 → bm1 versus cdd → bm1, *$P < .05$ by Mann-Whitney *U* test. (B) TNF α and IFN γ production by donor CD8⁺ T cells was determined by intracytoplasmic staining. *$P < .05$ by Mann-Whitney *U* test. (C) Overall pathologic damage of liver specimens correlated to the intensity of cell infiltration in portal triads, bile ducts/ductules, vascular, and hepatocellular areas. $P < .01$ by linear regression analysis. Symbols are same as in 3A. Error bars indicate mean ± SD.

more than wt $CD8^+$ T cells after transfer to allogeneic mice even though most $pfp^{-/-}$ $CD8^+$ T cells are eliminated in the contraction phase.[28] Unlike Fas-mediated AICD in $CD4^+$ T cells, the role of Fas/FasL on the homeostasis of $CD8^+$ T cells appears to be minimal.[29-31] The absence of both pathways leads to the unrestrained expansion of $CD8^+$ T cells, causing more severe GVHD. Although the involvement of IFN-γ and/or TNF-α in homeostasis in $CD8^+$ T cells after BMT remains to be determined,[32] these effects appear to be overshadowed by the 2 cytotoxic pathways. These findings support the findings of Marks et al, demonstrating that these cytolytic molecules must be present in donor $CD8^+$ T cells to regulate the contraction of alloreactive $CD8^+$ T cells after transplantation (L. Marks, E. R. Podack, R.B.L., Donor T cell contraction following MHC-mismatched allogeneic bone marrow transplantation requires CD8 mediated perforin and FasL dependent regulation, manuscript submitted).

The current results sharply contrast with the study by Braun et al[26] demonstrating that the absence of either pathway in donor T cells reduced GVHD and that the absence of both pathways completely abrogated GVHD lethality. That study used a nonmyeloablative conditioning regimen and demonstrated the important role of host immune cells containing perforin to prevent donor T cell expansion critical to the induction of GVHD.[6,26] The use of a myeloablative regimen in the current study probably reduced the host-versus-graft response to an insignificant level, magnifying the role of the cytotoxic pathways in AICD.

Perforin deficiency could enhance $CD8^+$ T cell expansion through decreased killing of APCs, resulting in prolonged stimulation of additional naive T cells.[33,34] Therefore, we determined whether the absence of cytolytic effector function permitted longer survival of host DCs, resulting in greater stimulation of donor T cells. Host DCs were not detected, however, in either the spleen, BM, or gut of any recipients of $CD8^+$ T cells on day 6 after BMT (data not shown). Recently, Merad et al[35] demonstrated that donor T cells eliminated host Langerhans cells (LCs) through a FasL-dependent cytolytic mechanism and that persistent host LCs could stimulate skin GVHD. Although host LCs might persist and play a role in skin GVHD in cdd recipients, our data suggest that the residual skin LCs are not responsible for the unrestrained expansion of only cdd cells because no such expansion was observed in recipients of gld cells (Figure 3A). In addition, we created BM chimeras (bm1 into B6) that would express allogeneic MHC class I only on BM-derived APCs and not on target epithelial cells, as described in a previous study.[4] B6 cdd T cells induced lethal GVHD in these chimeras (mean survival time [MST]: day 30) similar to wt bm1 recipients, confirming that persistent alloantigen expression on host cells did not account for unrestrained expansion of cdd cells.

In summary, our data suggest that in the $CD8^+$-mediated GVHD, the lack of both perforin and FasL impaired AICD, causing unrestrained expansion of $CD8^+$ T cells and lethal immunopathology of GVHD.

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Early Immune Reaction after Reduced-Intensity Cord-Blood Transplantation for Adult Patients

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Background. To investigate immune reactions after reduced-intensity cord-blood transplantation (RI-CBT).
Materials and Methods. We reviewed medical records of 57 adult RI-CBT recipients. Preparative regimen comprised fludarabine, total-body irradiation, and either melphalan (n=51) or busulfan (n=6). Graft-versus-host disease (GvHD) prophylaxis was cyclosporine. PostRI-CBT immune reactions were classified according to time course: pre-engraftment immune reactions (PIR), engraftment syndrome (ES), and GvHD.
Results. Forty-five patients achieved engraftment at a median of day 19. PIR was characterized by high-grade fever and weight gain and developed on a median of day 9 in 35 of the 45 evaluable patients, including 3 who did not achieve engraftment. PIR subsided spontaneously in 12 patients, whereas corticosteroids were required in the other 23. ES and grade I to IV acute GvHD developed in 36 and 29 patients, respectively. GvHD could not be distinguished from preceding PIR or ES in 10 patients. Causes of the 32 nonrelapse mortalities included GvHD (n=5) and PIR (n=1). There were no significant differences in relapse and nonrelapse deaths between patients with PIR and those without it (18% vs. 5%, and 60% vs. 65%, respectively).
Conclusions. Immune reactions after RI-CBT can be categorized into three distinct subtypes.

Keywords: Graft-versus-host disease, Engraftment syndrome, Preengraftment immune reaction, Allogeneic hematopoietic stem-cell transplantation, Nonmyeloablative stem-cell transplantation.

(*Transplantation* 2005;80: 34–40)

Cord-blood transplantation (CBT) is a promising approach for patients with advanced hematologic malignancies who lack a suitable donor. Cord blood has many theoretic advantages as a stem-cell source. Hematopoietic progenitors from cord blood are enriched in primitive stem cells, producing in vivo long-term repopulating stem cells (*1*). Another advantage of cord blood is immaturity of immune function. Long-lasting unresponsiveness and lack of proliferation of cord-blood lymphocytes on rechallenge with alloantigen might lead to reduced incidence of graft-versus-host disease (GvHD) after CBT (*2*), whereas a graft-versus-leukemia (GvL) effect might be maintained owing to the presence of precursor T and natural killer cells (*3, 4*). The feasibility of related and unrelated CBT has been demonstrated for pediatric patients (*5–8*), and the technique has been successfully applied to adults (*9–12*). Moreover, in adult patients with advanced hematologic malignancies, the feasibility of CBT using reduced-intensity preparative regimens (reduced-intensity CBT [RI-CBT]) has been demonstrated by us and other researchers (*13–16*).

Several types of immune reactions have been reported after allogeneic stem-cell transplantation (allo-SCT). With the exception of acute GvHD (*6, 17–19*), little information is available regarding immune reactions after CBT. The incidence and severity of acute GvHD after unrelated CBT have been low compared with those after allo-SCT from a matched unrelated donor or a mismatched family donor, despite the infusion of human leukocyte antigen (HLA)-mismatched graft (*20*); however, the reported incidence of grade II to IV acute GvHD varies from 25% to 72% (*5, 7–9, 11, 21–24*). Sanz et al. (*10*) reported that 21 of 22 adult CBT recipients developed grade I to IV acute GvHD and that median time to development of GvHD was 9 (range 4–14) days. Considering that median time to neutrophil engraftment was 25.5 (range 14–64) days, the majority of patients developed acute GvHD before engraftment. Similar findings have been reported by other groups (*14*). The circumstances of these immune reactions appear different from those seen in conventional allo-SCT.

Different immune reactions may occur after RI-CBT, and we postulated that characterization of the clinical features of these reactions in relation to engraftment would be useful. We investigated clinical features of immune reactions in 57 patients who underwent RI-CBT at Toranomon Hospital.

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PATIENTS AND METHODS

Patients

Fifty-seven patients underwent RI-CBT at Toranomon Hospital between January 2002 and August 2003. Patient characteristics and transplantation procedures are shown in Table 1. All patients had hematologic disorders or solid tumors that were incurable with conventional treatments and were considered inappropriate for conventional allo-SCT because of the lack of an HLA-identical sibling or a suitable unrelated donor, age greater than 50 years, or organ dysfunction. All patients provided written informed consent in accordance with the requirements of the institutional review board.

Transplantation Procedures

Cord-blood units that were matched for four or more of six HLA antigens and contained at least 2.0×10^7 nucleated cells/kg of recipient body weight before freezing were used. Cord-blood units were not depleted of T lymphocytes.

The preparative regimen comprised fludarabine 25 mg/m^2 on days −7 to −3, melphalan 80 mg/m^2 on day −2 (n=51) or busulfan 4 mg/kg for 2 days (n=6), and 4 or 8 Gy total-body irradiation (TBI) in 2 fractions on day −1 (Table 1). Granulocyte colony stimulating factor at 300 μg/m^2/day was administered intravenously from day 1 until neutrophil engraftment. Laboratory data including C-reactive protein (CRP) were obtained three times a week.

For GvHD prophylaxis, a continuous infusion of cyclosporine at 3 mg/kg from day −1 until toleration of oral administration was administered. Acute GvHD was graded according to the established criteria (*25*). Patients with grade II to IV acute GvHD were given 0.5 to 2.0 mg/kg per day of methylprednisolone. Treatment of immune reactions other than GvHD was at physicians' discretion. Management of infections was reported previously (*13*).

Chimerism Analysis

Chimerism was assessed using fluorescent in situ hybridization in sex-mismatched donor-recipient pairs. In sex-matched pairs, polymerase chain reaction for variable numbers of tandem repeats was used with donor cells detected at a sensitivity of 10% (*26*). Whole-blood and CD3+ cell chimerism was assessed at the time of granulocyte engraftment. When engraftment was delayed, chimerism was assessed on day 30. For those who died before engraftment, chimerism was assessed at least once during life.

Definition of Engraftment and Immune Reactions

Engraftment was defined as white blood cell count greater than 1.0×10^9/L or absolute neutrophil count greater than 0.5×10^9/L for 2 consecutive days. Graft failure was defined as peripheral cytopenia and marrow hypoplasia occurring later than day 60, accompanied by failure to detect donor markers using cytogenetic or molecular techniques.

The reported clinical presentation of engraftment or pre-engraftment syndrome (ES) varies, primarily because of the lack of uniform diagnostic criteria (*27*). When patients with no evidence of infection or adverse effects of medication exhibited skin eruption, diarrhea, jaundice (serum levels of

TABLE 1. Characteristics of patients receiving cord-blood transplantation

Characteristics	n=57
Age (yr)	
Median	56
Range	21–72
Sex	
Male	32
Female	25
Diagnosis	no. of patients
Cancer	
Acute lymphoblastic leukemia	
1st complete remission	1
Advanced disease	7
Acute myeloblastic leukemia	
1st complete remission	1
Advanced disease	20
Myelodysplastic syndrome	
Refractory anemia	1
Others	2
Chronic myeloid leukemia	
Advanced disease	1
Malignant lymphoma	
1st complete remission	1
Advanced disease	16
Multiple myeloma	
Advanced disease	1
Solid tumor	2
Bone marrow failure syndrome	
Severe aplastic anemia	4
Conditioning regimen	
Fludarabine/Busulfan/Total body irradiation 4 Gy	5
Fludarabine/Busulfan/Total body irradiation 8 Gy	1
Fludarabine/Melphalan/Total body irradiation 4 Gy	48
Fludarabine/Melphalan/Total body irradiation 8 Gy	3
Graft-versus-host disease prophylaxis	
Cyclosporin	57
Infused CD34+ cells ($\times10^5$/kg)	
Dose	2.9
Range	2.1–4.4
Body weight (kg)	
Median	53.8
Range	37.3–77.4
No. of HLA-A, B, and DRB1 mismatches	
0	1
1	8
2	48

HLA, human leukocyte antigen.