These preliminary results support the hypothesis that MMF can be used safely and has encouraging efficacy in the treatment of patients with GVHD who fail to benefit from conventional therapy. We emphasize that our results may have been influenced by the small number of patients in this study, and it is difficult to draw a final conclusion. In addition, MMF reduced the requirement for steroids, thereby reducing the risk of complications due to iatrogenic immunosuppression. A prospective randomized clinical trial is warranted to assess the impact of MMF in the treatment of refractory GVHD. The early combination of MMF with other treatment strategies may further improve the response rate and survival of these patients. Additional studies are also needed to test this hypothesis.

Acknowledgments

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Graft-Versus-Host Disease

Influence of transplanted dose of CD56+ cells on development of graft-versus-host disease in patients receiving G-CSF-mobilized peripheral blood progenitor cells from HLA-identical sibling donors

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Summary:

We investigated effects of variations in the cellular composition of G-CSF-mobilized peripheral blood progenitor cell (G-PBPC) allografts on clinical outcomes of allogeneic PBPC transplantation. We retrospectively analyzed transplanted doses of various immunocompetent cells from 27 HLA-identical sibling donors in relation to engraftment, incidence of graft-versus-host disease (GVHD), and survival. Significant variability was documented in both absolute numbers and relative proportions of CD34+, CD2+, CD3+, CD4^{high}+, CD4+25+, CD8^{high}+, CD19+, CD56+, and CD56+16+ cells contained in these allografts. Stepwise Cox regression analysis revealed that the CD56+ cell dose was significantly inversely correlated with the incidence of GVHD. Thus, there was a significantly higher incidence of grade II acute GVHD in patients receiving a lower CD56+16+ cell dose (hazard ratio (HR) 0.0090; 95% confidence interval (CI), <0.00001–3.38; $P=0.031$), a higher incidence of chronic GVHD in those receiving allografts with a lower CD56+16+ to CD34+ ratio (HR <0.00001; 95% CI <0.00001–0.0007; $P=0.0035$), and a higher incidence of extensive chronic GVHD in those receiving allografts with a lower CD56+ to CD34+ ratio (HR <0.00001; 95% CI <0.00001–0.053; $P=0.0083$). These results suggest that CD56+ cells in G-PBPC allografts from HLA-identical sibling donors may play an important role in preventing the development of GVHD.
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Keywords: CD56+ cells; G-CSF-mobilized peripheral blood progenitor cells; HLA-identical sibling donors; allogeneic peripheral blood; progenitor cell transplantation; graft-versus-host disease

G-CSF-mobilized peripheral blood progenitor cells (G-PBPC) from healthy donors have been used extensively as an alternative to bone marrow cells (BMC) in allogeneic transplantation for treatment of various hematologic malignancies.[1] Several reports indicate that clinical results of stem cell transplantation from HLA-identical sibling donors are improved by substituting G-PBPC for BMC.[2–8] The biological basis for the improvement of survival observed after allogeneic PBPC transplantation (allo-PBPCT) is not clear, but is likely to be related to differences in the cellular composition of G-PBPC and BMC allografts.[9–11]

In general, engraftment of neutrophils and platelets after allo-PBPCT is faster than after allogeneic bone marrow transplantation (allo-BMT). This difference is considered to be due to the larger dose of CD34+ progenitor cells in G-PBPC allografts compared to BMC. A close relation between the dose of CD34+ cells infused and rapid engraftment has also been shown in the setting of autologous stem cell transplantation.[12] G-PBPC allografts also contain 10-fold more CD3+ cells, 50-fold more CD14+ cells, and 19-fold more CD56+ natural killer (NK) cells than BMC, which may affect engraftment, development of graft-versus-host disease (GVHD), and survival.[5,13–15] There have been many reports concerning acute and chronic GVHD in prospective randomized trials comparing allo-PBSCT with allo-BMT,[4,5,7,16–21] but it is not known whether the incidence of acute and chronic GVHD is associated with differences in the cellular composition between G-PBPC and BMC allografts.

We therefore analyzed the relations between the cellular composition of G-PBPC allografts and clinical outcomes of

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allo-PBPCT in 27 adult Japanese patients with hematologic malignancies. In this study, the influences of absolute numbers and relative proportions of CD34+, CD2+, CD3+, CD4^{high}+, CD4+CD25+, CD8^{high}+, CD19+, CD56+, and CD56+16+ cells in G-PBPC allografts on engraftment, acute and chronic GVHD, and survival were investigated.

Materials and methods

Patients

This retrospective analysis included 27 patients who underwent allo-PBPCT from an HLA-identical sibling donor for different hematologic malignancies between March 1996 and May 2001. The results of transplantation were collected from medical records at four participating centers. All results were checked and confirmed by communication with one or more of the investigators at the transplant centers. The median follow-up time was 38.2 months (range 14.4–66.6 months) after transplantation. A total of 16 and 11 patients were categorized into standard-risk and high-risk groups, respectively. The standard-risk group included acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) in first complete remission (CR), chronic myeloid leukemia (CML) in first chronic phase (CP) and refractory anemia (RA) of myelodysplastic syndrome (MDS). The high-risk group included AML in second CR, CML in second CP, MDS in RA with excess of blasts (RAEB), RAEB in transformation (RAEB-t), transformation to AML (tAML), and more advanced status of disease.

Donors

Donor selection was based on serological typing for HLA-A, HLA-B, and HLA-DR antigens. G-CSF (filgrastim, Kirin Brewery/Sankyo Co., Tokyo, Japan) was administered subcutaneously to donors at a dose of 400 μg/m^2 per day for 4 or 5 days as previously reported.[22] Leukaphereses were performed using a continuous-flow blood cell separator (Cobe Spectra, Cobe Laboratories, Lakewood, CO, USA) for 1–3 days beginning on day 4 of G-CSF administration until 3.0×10^6 CD34 cells/kg (patient body weight) had been collected.[22] This target dose was achieved in all but one of the donors (4%) by performing two or more leukaphereses. All G-PBPC collected were infused without cryopreservation on either day 0, day 1, or day 2.

Immunophenotyping of G-PBPC

Immunophenotyping of CD34+, CD2+, CD3+, CD4^{high}+, CD4+CD25+, CD8^{high}+, CD19+, CD56+, and CD56+16+ cells in each G-PBPC allografts was performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) at the Cancer Center, Kyushu University Hospital. For each sample, seven tubes were prepared and processed in parallel according to the following protocols: (1) single staining with CD34 fluorescein isothiocyanate (FITC) (HPCA-2-FITC, Becton Dickinson) and (2) matched isotype controls (Becton Dickinson). Viability of G-PBPC in the tubes prepared according to the protocols was determined by staining with 7-aminoactinomycin-D (7-AAD). Gates were set around forward *vs* side scatter, and low 7-AAD staining was used to include only viable nucleated cells for further analysis. In addition, two-color FCM was also performed as follows: (1) double staining with CD3 phycoerythrin (PE) (Leu-4, Becton Dickinson) and CD19 FITC (Leu-12, Becton Dickinson), (2) double staining with CD4 PE (Leu-3a, Becton Dickinson) and CD25 FITC (IL-2R, Becton Dickinson), (3) double staining with CD8 PE (Leu-2a, Becton Dickinson) and CD2 FITC (Leu-5b, Becton Dickinson), (4) double staining with CD56 PE (Leu-19, Becton Dickinson) and CD16 FITC (Leu-11a, Becton Dickinson), and (5) matched isotype controls (Becton Dickinson). Gates were set around forward *vs* side scatter to include only viable nucleated cells for further analysis.

Transplant procedure and supportive care

Patients were conditioned with myeloablative regimens as described in Table 1. Cyclosporine (CYA) and methotrexate (MTX) were given for standard GVHD prophylaxis. CYA was instituted intravenously from day −1 at a dose of 3 ($n = 5$) or 5 mg/kg ($n = 22$) and doses were adjusted to maintain whole blood levels at 200–300 ng/ml. MTX was infused at 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6 (or 7) after transplantation. Grade II acute GVHD was treated with prednisolone according to a standard regimen.[23] In the absence of acute or chronic GVHD, CYA administration was tapered off by 12 months after transplantation, whereas it was continued in patients developing chronic GVHD.

Table 1 Patient, donor, and treatment characteristics ($n = 27$)

Median age, years (range)	
Patients	48 (26–56)
Donors	45 (23–64)
Gender (no. of patients)	
Patients: female/male	14/13
Donors: female/male	14/13
Diagnosis (No. of patients)	
Acute myeloid leukemia (AML)	8
Acute lymphoblastic leukemia (ALL)	5
Chronic myeloid leukemia (CML)	5
Myelodysplastic syndrome (MDS)	5
Non-Hodgkin's lymphoma (NHL)	4
Disease status	
Standard-risk	11
High-risk	16
Conditioning regimen[a]	
TBI + CY ± others	16
BU + CY ± others	11
G-CSF administration after transplantation	
Yes	25
No	2

[a]TBI = total body irradiation (12 or 13.2 Gy), CY = cyclophosphamide (120 or 150 mg/kg), BU = busulfan (16 mg/kg), others = Ara-C, VP-16, or thioTEPA.

Engraftment

Engraftment was defined as an absolute neutrophil count (ANC) exceeding 500/μl for three consecutive days after allo-PBPCT. The day of engraftment was determined to be the first of these three consecutive days. Platelet recovery was defined as a platelet count exceeding 20×10^9/l without platelet support. Graft failure was defined as an ANC never exceeding 0.5×10^9/l or failing to maintain more than 0.5×10^9/l for at least three consecutive days by day 28.

Clinical outcomes

The severity of acute GVHD was graded according to the consensus criteria.[24] Chronic GVHD was assessed and graded according to the standard criteria.[25] Progression-free survival (PFS) was defined as time interval from transplantation to the first event such as relapse, progressive disease, or death. Relapse of the disease was defined as recurrence of the disease including the reappearance of Philadelphia chromosome in CML. Overall survival (OS) was defined as the time between transplantation and death.

Statistical analysis

In order to assess associations of the cellular composition of allografts with the time to neutrophil and platelet engraftment and with the incidence of acute GVHD, we examined variables such as patient and donor age, gender, and disease status. When we analyzed associations of the cellular composition of allografts with the incidence of chronic GVHD, PFS, and OS, we examined variables such as patient and donor age, gender, disease status, and the incidence of acute GVHD. The stepwise Cox regression model for multivariate analysis was used to evaluate these associations. *P*-values of 0.05 or less were considered to indicate statistical significance. Cumulative incidence estimates were used to show associations of CD56+16+ cell doses with the incidence of grade II acute GVHD, CD56+16+ to CD34+ ratios with chronic GVHD, and CD56+ to CD34+ ratios with extensive chronic GVHD.[26] Statistical analysis was performed with SAS ver 6.07.

Results

Cellular composition of G-PBPC allografts

Table 2 shows absolute numbers of CD34+, CD2+, CD3+, $CD4^{high}$+, CD4+25+, $CD8^{high}$+, CD19+, CD56+, and CD56+16+ cells in G-PBPC allografts. Both their absolute numbers and relative proportions varied significantly. Median numbers of CD34+, CD3+, $CD4^{high}$+, $CD8^{high}$+, CD56+, and CD56+16+ cells were 9.4×10^6/kg, 5.9×10^8/kg, 3.5×10^8/kg, 2.4×10^8/kg, 7.7×10^7/kg, and 6.0×10^7/kg, respectively.

Engraftment

No graft failure occurred in patients undergoing allo-PBPCT. The median time to neutrophil engraftment was 12 days (range 10–21 days). A total of 25 patients (93%) achieved platelet engraftment. The median time to platelet engraftment was 17 days (range 10–50 days). There were no significant associations of absolute numbers or relative proportions of CD34+, CD2+, CD3+, $CD4^{high}$+, CD4+CD25+, $CD8^{high}$+, CD19+, CD56+, or CD56+16+ cells in G-PBPC allografts with the number of days required for engraftment.

Acute and chronic GVHD

The incidence, severity, and onset of acute and chronic GVHD are shown in Table 3. Grade II acute GVHD developed in six patients (22%); severe acute GVHD (grade III or IV) was not observed. There was a significantly higher incidence of grade II acute GVHD in patients receiving a lower CD56+16+ cell dose (hazard ratio (HR) 0.0090; 95% confidence interval (CI) <0.00001–3.38; $P = 0.031$) (Table 4, Figure 1). There were no significant relations between absolute numbers of CD34+, CD2+, CD3+, $CD4^{high}$+, CD4+CD25+, $CD8^{high}$+, CD19+, and CD56+ cells or relative proportions of CD34+, CD2+, CD3+, $CD4^{high}$+, CD4+CD25+, $CD8^{high}$+, CD19+, CD56+, and CD56+16+ cells and the incidence of grade II acute GVHD.

Table 2 Cellular composition of G-PBPC allografts

	Median cell number (range)
CD34+ cells ($\times 10^6$/kg)[a]	9.4 (1.2–35.4)
CD2+ cells ($\times 10^8$/kg)	6.5 (2.0–17.3)
CD3+ cells ($\times 10^8$/kg)	5.9 (1.8–15.9)
$CD4^{high}$ +cells ($\times 10^8$/kg)	3.5 (1.1–10.2)
CD4+25+ cells ($\times 10^7$/kg)	5.2 (1.4–17.8)
$CD8^{high}$ +cells ($\times 10^8$/kg)	2.4 (0.7–6.2)
CD19+ cells ($\times 10^8$/kg)	1.4 (0.4–4.5)
CD56+ cells ($\times 10^7$/kg)	7.7 (1.1–20.4)
CD56+16+ cells ($\times 10^7$/kg)	6.0 (0.9–19.2)

[a]/kg = per kg patient body weight.

Table 3 Incidence of acute GVHD and chronic GVHD

	No. of patients (%)	*Median onset, days (range)*
Acute GVHD		
0	13 (48%)	—
I	8 (30%)	29 (3–67)
II	6 (22%)	20 (13–25)
III, IV	0 (0%)	—
Chronic GVHD		
None	15 (55%)	—
Limited	4 (15%)	190 (100–281)
Extensive	8 (30%)	111 (100–282)
Organs involved		
Liver	9 (75%)	120 (100–260)
Skin	6 (50%)	101 (100–281)
Mouth	5 (42%)	120 (100–260)
Eye	4 (33%)	111 (100–186)
Lung	3 (25%)	186 (100–282)
Gastrointestinal tract	1 (8%)	186

Table 4 Summary of stepwise Cox regression analysis

Parameter[a]	*Variable*	*Coefficient*	*s.e.*	*HR*[b]	*95% CI*[c]	*P-value*
Grade II acute GVHD	CD56+16+ cell dose	−4.712	3.026	0.009	<0.00001–3.380	0.0312
Chronic GVHD						
Limited and extensive	CD56+16+ to CD34+ ratio	−31.238	12.226	<0.00001	<0.00001–0.0007	0.0035
Extensive	CD56+ to CD34+ ratio	−37.119	17.442	<0.00001	<0.00001–0.0532	0.0083

[a]s.e. = standard error, [b]HR = hazard ratio, [c]CI = confidence interval.

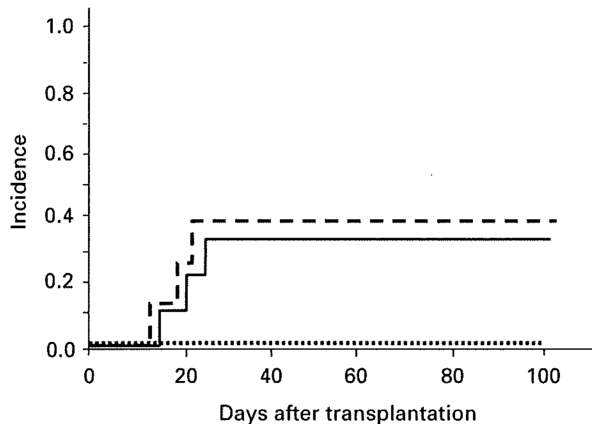

Figure 1 , Grade II acute GVHD is associated with CD56+16+ cell dose. The incidence of grade II acute GVHD after allo-PBPCT from HLA-identical siblings is increased in patients receiving a lower CD56+16+ cell dose: 0.9–3.0 × 10^7/kg (*n* = 9, solid line), 3.1–7.0 × 10^7/kg (*n* = 8, dashed line), and 7.1–19.2 × 10^7/kg (*n* = 10, dotted line).

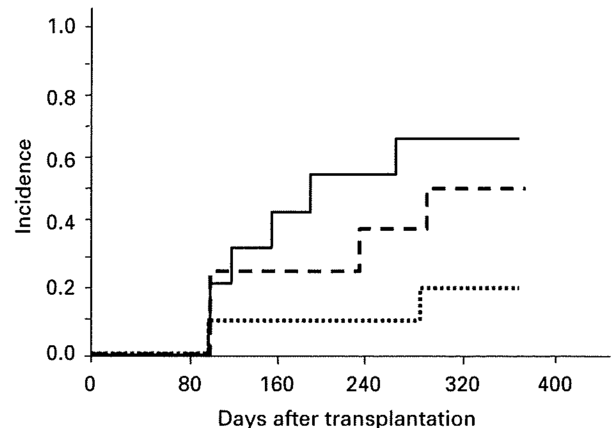

Figure 2 Chronic GVHD is associated with CD56+16+ to CD34+ ratios. The incidence of chronic GVHD after allo-PBPCT from HLA-identical siblings is increased in patients receiving allografts with a lower CD56+16+ to CD34+ ratio: 1 to 3-fold (*n* = 9, solid line), 4 to 7-fold (*n* = 8, dashed line), and 8 to 23-fold (*n* = 10, dotted line).

Limited and extensive forms of chronic GVHD developed in four (15%) and eight (30%) patients, respectively. The liver was predominantly affected in chronic GVHD, especially in extensive chronic GVHD. An increased incidence of chronic GVHD was observed in patients who received G-PBPC allografts characterized by a lower CD56+16+ to CD34+ ratio (HR <0.00001; 95% CI <0.00001–0.0007; $P = 0.0035$) (Figure 2). In addition, a significantly increased incidence of extensive chronic GVHD was observed in patients who received G-PBPC allografts with a lower CD56+ to CD34+ ratio (HR <0.00001; 95% CI <0.00001–0.053; $P = 0.0083$) (Figure 3). When chronic GVHD was analyzed, no significant associations were found between transplanted doses of CD34+, CD56+, and CD56+16+ cells and chronic GVHD.

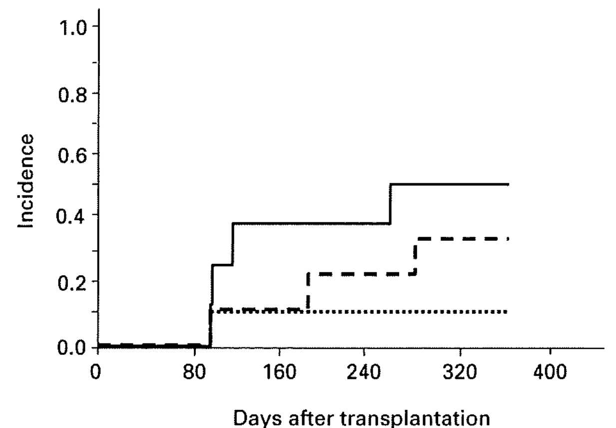

Figure 3 Extensive chronic GVHD is associated with CD56+ to CD34+ ratios. The incidence of extensive chronic GVHD after allo-PBPCT from HLA-identical siblings is increased in patients receiving allografts with a lower CD56+ to CD34+ ratio: 1 to 4-fold (*n* = 8, solid line), 5 to 10-fold (*n* = 9, dashed line), and 11 to 61-fold (*n* = 10, dotted line).

Cause of death, relapse, and survival

Five patients died after transplantation; three of them relapsed or progressed, and the remaining two died from extensive chronic GVHD. Probabilities of PFS and OS at 3 years after transplantation were 81.3 and 86.5% in the standard-risk group, and 62.3 and 71.6% in the high-risk group, respectively. There were no significant associations between absolute numbers or relative proportions of CD34+, CD2+, CD3+, $CD4^{high}$+, CD4+CD25+, $CD8^{high}$+, CD19+, CD56+, or CD56+16+ cells and either PFS or OS.

Discussion

The numbers and proportions of different types of cells contained in G-PBPC allografts were found to vary greatly among donors. Although this variability may be caused by different factors in G-CSF-induced mobilization from healthy donors, differences in numbers of CD34+, CD2+, CD3+, $CD4^{high}$+, CD4+25+, $CD8^{high}$+, CD19+, CD56+, and

CD56+16+ cells in G-PBPC allografts were analyzed in relation to clinical outcomes after allo-PBPCT from an HLA-identical sibling donor. Our findings indicated that a transplanted dose of CD56+ cells was significantly inversely correlated with the incidence of GVHD.

There was no significant difference between the time to engraftment and absolute numbers or relative proportions of CD34+, CD2+, CD3+, $CD4^{high}$+, CD4+CD25+, $CD8^{high}$+, CD19+, CD56+, or CD56+16+ cells in G-PBPC allografts. Zaucha *et al*[27] demonstrated in a retrospective study that there was a relatively weak association of transplanted dose of CD34+ cells in G-PBPCT allografts from HLA-identical sibling donors with the time to neutrophil engraftment. The reason for the lack of associations found here may be due to the small number of patients analyzed in our study.

It has been demonstrated that T cells such as CD3+ cells, $CD4^{high}$+ cells, and $CD8^{high}$+ cells in the graft play a crucial role in the development of acute and chronic GVHD.[28,29] Clinical results indicate that depletion of T cells with anti-CD3 antibody effectively prevents acute GVHD.[30] However, in the present study, there was no significant association of transplanted doses of CD3+ cells with development of grade II acute GVHD, in agreement with the report of Zaucha *et al*[27]

In the current study, there was a significantly greater incidence of grade II acute GVHD in patients who received G-PBPC allografts containing a lower dose of CD56+16+ cells, especially if this was less than 7.0×10^7/kg. Ruggeri *et al*[31] reported that donor NK cells could suppress leukemia relapse and graft rejection as well as protect patients from GVHD. Their results showed that alloreactive NK cells in major-histocompatibility (MHC)-mismatched allo-BMT eradicated human leukemia cells, killed host lymphohematopoietic cells, and reduced GVHD by eliminating recipient-type antigen presenting cells. In a murine allo-BMT model, moreover, cloned T-cell lines expressing NK1.1 (a murine NK cell marker) have been reported to protect against GVHD.[32,33] Other studies in mice have confirmed that this protection against GVHD is lost after depletion of cells bearing asialo GM1, an NK cell marker.[34] These results suggest that NK cells may play an important role in controlling GVHD and support our findings that double-positive CD56+16+ NK cells might affect grade II acute GVHD, although the number of patients analyzed here was small. A further study to clarify the role of NK cells in allo-PBPCT may be warranted.

In our study, no patients developed grade III and IV acute GVHD. Moreover, five of six patients, who developed grade II acute GVHD, responded well to treatment with prednisolone, suggesting that a combination of CYA and MTX may be optimal for GVHD prophylaxis after allo-PBPCT from HLA-identical sibling donors regardless of the number or type of cells in G-PBPC allografts. In addition to analyzing ethnically different populations, differences in GVHD prophylaxis regimens may explain discrepancies in the incidence of acute GVHD reported in other studies.[27,35]

In the present study, there was a significantly higher incidence of chronic GVHD in patients who received G-PBPC allografts characterized by a lower CD56+16+ to CD34+ ratio, especially when this ratio was seven-fold lower, and also a higher incidence of extensive chronic GVHD in those who received G-PBPC allografts characterized by a lower CD56+ to CD34+ ratio, especially when this ratio was 10-fold lower. However, the association of transplanted doses of CD34+, CD56+, and CD56+16+ cells with chronic GVHD was not statistically significant. NK cells, NKT cells, and precursor cells of T and B cell lineages express surface CD56. This result suggests that patients who received G-PBPC allografts characterized by a lower CD56+16+ to CD34+ ratio and CD56+ to CD34+ ratio may not have received a sufficient dose of double-positive CD56+16+ NK cells or CD34+ cells, together with which dendritic cells might have been transferred, to suppress donor T-cell proliferation.[36] Moreover, to control extensive chronic GVHD, we may also need to transplant a suitable dose of CD56+ cells including CD56+16+ cells as well as CD34+ cells for successful allo-PBPCT. Our result stands in contrast to those reported by Zaucha *et al*[27] and Przepiorka *et al*[37] In their study, most of all G-PBPC collected were probably infused with cryopreservation. Moreover, in the study reported by Przepiorka *et al*, GVHD prophylaxis regimens were different from ours. Therefore, the differences in G-PBPC infused with cryopreservation or those in GVHD prophylaxis regimens may explain the discrepancies between these two studies and ours.

In conclusion, the present study suggests that double-positive CD56+16+ cells contained in G-PBPC allografts from HLA-identical sibling donors may play an important role in preventing the development of acute GVHD. Moreover, CD56+ cells, especially double-positive CD56+16+ cells, together with an appropriate dose of CD34+ cells, may also influence the development of chronic GVHD. Although further studies of G-PBPC allografts are needed to determine whether cell separation approaches can be applied to allo-PBPCT from HLA-identical sibling donors, our data suggest that technological advances in cell separation of G-PBPC allografts, especially CD56+ cells and CD34+ cells, may influence the development of acute and chronic GVHD after allo-PBPCT.

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Unrelated donor transplants

Allogeneic peripheral blood stem cell transplantation from two- or three-loci-mismatched related donors in adult Japanese patients with high-risk hematologic malignancies

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Summary:

With the increasing frequency of haploidentical transplantation, it is becoming more important to establish the degree of HLA mismatch that can be accepted. We retrospectively analyzed clinical data of 50 adult Japanese patients with high-risk hematologic malignancies who underwent allogeneic peripheral blood stem cell transplantation (PBSCT) from two- or three-loci-mismatched related donors with HLA class I and II gene disparities in the graft-versus-host direction. They were treated at 20 transplant centers between 1996 and 2002. In all, 18 patients received unmanipulated PBSC, while 32 received purified CD34+ blood cells. Conventional ($n=31$) or reduced-intensity ($n=19$) conditioning regimens were used. Of the 39 patients (78%) who survived for ≥28 days after transplant, 37 (95%) achieved neutrophil engraftment, while graft failure and rejection occurred in two of 39 (5%) and three of 37 (8%) patients, respectively. Stepwise Cox regression analysis revealed a significantly lower incidence of grades II–IV acute GVHD in patients receiving purified CD34+ cells (hazard ratio 0.32; 95% CI 0.12–0.84; $P=0.022$). By 1 year post transplant, 28 patients (56%) had died of transplant-related problems, including infectious complications (30%). Although the number of patients is small, our data suggest that transplant-related problems, particularly infectious complications, are major obstacles to the success of this therapy.

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Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapeutic approach for a number of life-threatening hematologic malignancies.[1] Unfortunately, only 30–40% of patients have a matched related donor (MRD) or a one-locus-mismatched related donor (MMRD), mismatched for either HLA-A, -B or -DR antigens, availabe.[2] Therefore, most transplant centers perform allogeneic HSCT from a matched unrelated donor (MUD) as a second option for those who do not have an MRD. Even though a large number of volunteers are willing to donate bone marrow, there are candidate patients for whom an MUD is not available. Moreover, the aggressive nature of their diseases often precludes a lengthy search for an MUD. Recently, unrelated cord blood (CB) has been used as a source of allogeneic HSCT.[3] However, in some cases, it is difficult to collect a sufficient number of stem cells from CB to engraft adult patients, which has been reported as an important factor of improved event-free survival.[4] Therefore, two- or three-loci-MMRD, which are readily available, have been proposed as a potential stem cell source.

The degree of HLA disparity between patients and donors has been reported to have a major impact on the outcome of allogeneic HSCT, particularly on engraftment and acute GVHD.[5,6] Although the incidence of graft failure

in BMT from a three-loci-MMRD or a haploidentical related donor has been reported to be higher than in BMT from a one-locus- or two-loci-MMRD,[7] allogeneic HSCT from a two- or three-loci-MMRD was demonstrated to be feasible for the achievement of stable engraftment.[8] Allogeneic HSCT from a two-loci-MMRD was reported to be associated with a similar incidence and severity of acute GVHD as conventional allogeneic HSCT from a one-locus-MMRD,[9] especially in patients receiving purified CD34+ blood cells.[10] It is becoming more important to establish the degree of HLA mismatch that can be accepted.

The Japanese population is believed to be relatively homogeneous both ethnically and genetically, with less HLA genetic diversity than Caucasians.[11,12] This could make unmanipulated allogeneic HSCT between two- or three-loci-mismatched pairs more feasible in this population because there may be lower incidence of graft failure and GVHD. Allogeneic peripheral blood stem cell transplantation (PBSCT) is increasingly undertaken in Japan since it became eligible for reimbursement from health insurance in the year 2000. We conducted a nationwide retrospective survey to investigate the time course of engraftment, incidence of acute and chronic GVHD, transplant-related problems and survival in patients undergoing allogeneic PBSCT from two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the graft-versus-host (GVH) direction.

Patients and methods

Patient characteristics

This study included 50 Japanese patients over age 16 who underwent allogeneic PBSCT from two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the GVH direction for a variety of high-risk hematologic malignancies. They were transplanted between January 1996 and March 2002 at 20 different transplant centers in Japan. The patients consisted of 17 (34%) with AML, six (12%) with ALL, six (12%) with myelodysplastic syndrome (MDS), eight (16%) with CML, 11 (22%) with non-Hodgkin's lymphoma (NHL) and two (4%) with multiple myeloma (MM). All of them were considered to be at high risk for relapse or to be refractory to intensive chemotherapy: 18 patients (36%) were transplanted at primary refractory status, 17 (34%) at chemorefractory status, nine (18%) in relapse after prior autologous PBSCT, three (6%) in relapse after prior allogeneic BMT from an MUD (MUD-BMT), one (2%) in relapse after prior unrelated CB transplantation and MUD-BMT, and two (4%) after graft rejection following MUD-BMT for MDS. A total of 33 patients (66%) underwent primary allogeneic PBSCT and 17 (34%) underwent allogeneic PBSCT after prior autologous PBSCT ($n = 10$, 20%) or prior allogeneic HSCT ($n = 7$, 14%). The median time interval between prior autologous PBSCT or allogeneic HSCT and this therapy was 10.8 (range 3.1–27.3) or 5.3 months (1.7–29.2 months), respectively. Patient characteristics are summarized in Table 1. Treatment protocols were approved by local institutional review boards and all patients provided informed consent.

Stem cell collection and graft manipulation

G-CSF (Filgrastim, Kirin Brewery/Sankyo Co., Tokyo, Japan) was administered subcutaneously to donors at a dose of 400 $\mu g/m^2$ per day for 4 or 5 days as previously reported.[13] Leukaphereses were performed using a continuous-flow blood cell separator (Cobe Spectra, Cobe Laboratories, Lakewood, CO, USA) for 1–3 days beginning on day 4 of G-CSF administration until 3.0×10^6 CD34 cells/kg (patient body weight) had been collected. The percentages of CD34+ and CD3+ cells in the graft were determined by flow cytometry. Median doses of nucleated, CD34+ and CD3+ cells infused are shown in Table 1. Immunomagnetic selection was performed using a CliniMACS device ($n = 17$; Kirin Brewery, Tokyo, Japan)[14] or an Isolex system ($n = 15$; Baxter, Munich, Germany)[15] according to the manufacturer's recommendations. The graft was cryopreserved until infusion as previously reported.[16]

Conditioning regimen and GVHD prophylaxis

A conventional conditioning regimen was administered to 31 patients (62%). In all, 26 (52%) received 8–13.2 Gy TBI, and eight (16%) were given antithymocyte globulin (ATG) as an additional immunosuppressive. All patients treated with a conventional conditioning regimen received GVHD prophylaxis (CYA-based, $n = 18$; FK506-based, $n = 13$), as summarized in Table 2.

A total of 19 patients (38%) were treated with a reduced-intensity conditioning regimen: eight (16%) received 2–6 Gy TBI and 11 (22%) were given ATG. All but one of these patients treated with a reduced-intensity conditioning regimen received GVHD prophylaxis (CYA-based, $n = 8$; FK506-based, $n = 9$; prednisolone alone, $n = 1$).

Definition of outcome

Neutrophil engraftment was defined as an absolute neutrophil count (ANC) exceeding 0.5×10^9/l for 3 consecutive days after transplant. The day of neutrophil engraftment was determined to be the first of these 3 consecutive days. Platelet engraftment was defined as a platelet count exceeding 20×10^9/l without platelet support. In all 42 patients (84%) received G-CSF until neutrophil engraftment after transplant. If the ANC never exceeded 0.5×10^9/l or if it was not maintained above 0.5×10^9/l for at least 3 consecutive days by day 28 post transplant, the patient was considered to have 'graft failure'. If ANC of greater than 0.5×10^9/l could not be maintained after initial engraftment, the patient was considered to have 'graft rejection'.

The severity of acute GVHD was graded according to the consensus criteria[17] among 43 evaluated patients (86%) who developed acute GVHD within 28 days or who survived $\geqslant 28$ days after transplant. Chronic GVHD was assessed and graded according to the standard criteria[18]

Table 1 Patient, donor and graft characteristics

	Manipulation		
	No. (n = 18)	*CD34+ cell selection (n = 32)*	
		CliniMACS (n = 17)	*Isolex (n = 15)*
Median age (range) (years)			
Patients	32 (20–55)	35 (18–69)	29 (17–48)
Donors	42 (16–60)	42 (18–59)	44 (21–67)
Gender match, donor/patient			
Male/male	4[a]	8	8
Female/male	5	5	3
Female/female	8	2	2
Male/female	1	2	2
Relationship of donor to patient			
Father/mother	1/7	2/2	3/2
Sibling	5	9	9
Son/daughter	0/5	3/1	0/0
Aunt	0	0	1
Genotypic disparities			
GVH direction			
HLA-A/-B and -DRB1	1/9	1/7	1/3
HLA-A, -B and -DRB1	8	9	11
HVG direction			
HLA-A/-B/-DRB1	0/0/0	0/1/2	1/0/0
HLA-A and -B	0	1	1
HLA-A/-B and -DRB1	1/10	1/5	1/3
HLA-A, -B and -DRB1	7	7	9
Disease at transplant			
AML	2	9	6
ALL	1	2	3
MDS	4	0	2
CML	4	3	1
NHL	6	2	3
MM	1	1	0
Prior autologous PBSCT/allogeneic HSCT	3/4	2/3	5/0
Median time interval[b] (range) (months)	10.5 (0.2–24.2)	4.0 (0.4–96.8)	1.0 (0.1–38.1)
ECOG PS 0/1/2/3	3/8/5/2	5/8/3/1	6/7/1/1
Median graft size (range)[c]			
Nucleated cell dose ($\times 10^7$/kg)	78.2 (11.3–324.0)	0.71 (0.36–1.4)	0.38 (0.19–0.56)
CD34+ cell dose ($\times 10^6$/kg)	4.2 (1.5–9.5)	6.8 (2.9–13.5)	4.4 (0.67–9.8)
CD3+ cell dose (/kg)	2.7 (1.3-5.4) $\times 10^8$	2.8 (0.30–5.0) $\times 10^4$	4.0 (1.7–24.7) $\times 10^4$
Median follow-up (range) (months)	2.5 (0.10–15.4)	3.8 (0.20–16.8)	2.7 (0.30–35.5)

[a]Number of patients unless indicated otherwise.
[b]Time from diagnosis to transplant.
[c]The dose of CD34+ cells was significantly higher in patients receiving CD34+ blood cells purified by a CliniMACS device than in those receiving unmanipulated PBSC ($P = 0.0027$) or CD34+ blood cells purified by an Isolex system ($P = 0.014$). The dose of CD3+ cells was significantly higher in patients receiving unmanipulated PBSC than in those receiving CD34+ blood cells purified by a CliniMACS device ($P = 0.0004$) or an Isolex system ($P = 0.0009$).GVH, graft-versus-host; HVG, host-versus-graft; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; MM, multiple myeloma; ECOG PS, Eastern Cooperative Oncology Group performance score.

among 25 patients with sustained engraftment for ≥ 100 days after transplant.

Regimen-related toxicity (RRT) of organ systems before day 100 post transplant was graded according to the criteria proposed by Bearman *et al.*[19] A clinical diagnosis of veno-occlusive disease (VOD) was made based on the presence of jaundice (bilirubin ≥ 2 mg/dl), hepatomegaly and/or right upper quadrant pain, and $\geq 5\%$ weight gain from admission, with or without ascites.[20] The diagnosis of thrombotic microangiopathy (TMA) was made if the patient had thrombocytopenia (defined as a platelet count $< 100 \times 10^9$/l), microangiopathic hemolytic anemia as indicated by red blood cell fragmentation present in a peripheral blood smear and elevated lactate dehydrogenase (LDH), without an identifiable cause for the thrombocytopenia or microangiopathic hemolytic anemia (eg, sepsis, disseminated intravascular coagulation, carcinoma, eclampsia).[21]

Relapse was defined either by morphologic evidence of the disease in the peripheral blood, marrow or extramedullary sites, or by recurrence and sustained presence of pretransplant chromosomal abnormalities in cytogenetic analysis of the marrow cells. Patients with CML, in whom the sole evidence of the disease was positivity for the bcr/abl RNA transcript by polymerase chain reaction, were not classified as having relapse. Both relapse and progression were defined as disease progression with transplant-related deaths being censored. Treatment-related mortality (TRM)

Table 2 Treatment characteristics

	Manipulation		
	No. (n = 18)	*CD34+ cell selection (n = 32)*	
		CliniMACS (n = 17)	*Isolex (n = 15)*
Conventional conditioning regimen	7 (39%)[a]	9 (53%)	15 (100%)
TBI + CY + others[b]/TBI + melphalan	3/0	8/1	14/0
BU + CY + others[c]	4	0	1
ATG-containing	1 (6%)	0	7 (47%)
GVHD prophylaxis			
CYA + MTX/CYA + prednisolone/CYA	1/0/0	5/0/1	7/2/2
FK506 + MTX/FK506	6/0	3/0	0/4
Reduced-intensity conditioning regimen	11 (61%)	8 (47%)	0
TBI + CY/TBI + Flu + BU/TBI + Flu + ATG + others[d]/TBI + BU + ATG	1/0/0/0	0/2/4/1	0/0/0/0
Flu + others[e]	10	1	0
ATG-containing	6 (33%)	5 (29%)	0
GVHD prophylaxis			
CYA + MTX/CYA + prednisolone/CYA + MMF/CYA	1/1/0/1	0/0/3/2	0/0/0/0
FK506 + MTX/FK506 + prednisolone + MMF/FK506 + prednisolone/FK506	6/1/1/0	0/0/0/1	0/0/0/0
Prednisolone/none	0/0	1/1	0/0
G-CSF after transplant	12 (67%)	16 (94%)	14 (93%)

[a]Number of patients (%) unless indicated otherwise.
[b]Others = ATG, BU, Ara-C, thiotepa or VP-16.
[c]Others = ATG, Ara-C, Flu or melphalan.
[d]Others = BU, CY or thiotepa.
[e]Others = BU, CY, Ara-C, idarubicin or melphalan.
ATG, antithymocyte globulin; Flu, fludarabine; MMF, mycophenolate mofetil.

was defined as death from any cause other than relapse or disease progression. Progression-free survival (PFS) was defined as the time interval from transplant to the first event including relapse, disease progression and death. Overall survival (OS) was defined as the time interval from transplantation to death.

Data collection

Questionnaires were returned by 216 of 432 (50%) transplant centers in Japan.

A total of 91 adult patients underwent allogeneic HSCT from a genotyped related donor mismatched at two or three loci in the GVH direction for the treatment of hematologic malignancies in a total of 32 centers (listed in the Appendix).[22] These data were collected from medical records. Histocompatibility was determined by serology for HLA-A, -B and -DR antigens and/or by DNA typing for HLA-A, -B and -DRB1. All of these HLA data were reviewed, and inquiries concerning patient and donor HLA typings were verified by the central committee. Of these 91 patients, 24 were excluded because they received BM, five because they were thought not to be at high risk for relapse or refractory to intensive chemotherapy, and six because their donors were not anticipated to be serological two- or three-loci-MMRD. As in allogeneic HSCT from related as well as unrelated donors, both HLA class I and II gene disparities contribute to increased incidence of graft failure or acute GVHD prophylaxis,[23,24] identification of the fact that both HLA class I and II genes were disparate would be helpful. Thus, six patients were excluded because they received allogeneic PBSC from a two- or three-loci-MMRD with only HLA class I or II gene disparity in the GVH direction. Finally, we confirmed that 50 evaluable patients underwent allogeneic PBSCT from a two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the GVH direction for high-risk hematologic malignancies in 20 centers.

Statistical analysis

Comparisons of variables were performed using the two-tailed Fisher exact test or the χ^2 test. Continuous variables were compared by the Mann–Whitney U-test or the Kruskal–Wallis test. Competing risks for grades II–IV acute GVHD included death without grades II–IV acute GVHD, relapse, and graft failure and rejection. Relapse was a competing risk for TRM at day 100 post transplant, and TRM was a competing risk for relapse. The association of variables with the outcome was evaluated in multivariable analyses, with the use of stepwise Cox regression to adjust for differences in potentially confounding variables.[25–27] The variables considered were age and gender of patients or donors, number of transplantations, performance status (PS, which was evaluated before the start of the conditioning regimen and graded according to the Eastern Cooperative Oncology Group (ECOG) performance score), CD34 + cell doses, degree of HLA genotypic disparity in the GVH direction, conditioning regimens including reduced regimens, TBI and ATG used, GVHD

prophylaxis and G-CSF used after transplant. Each of these factors was checked for the assumption of proportional hazards by using a time-dependent covariate. The interactions with outcomes, such as the incidence of neutrophil and platelet engraftment, complications including infection before day 100 post transplant, grades II–IV acute GVHD and TRM at day 100 post transplant, were assessed in a model that included these outcomes and the variables under consideration. Only factors significantly associated with the outcomes ($P<0.05$) were retained in the final models. End points were calculated on the day of the last patient contact. The outcomes PFS and OS following transplant were estimated by the Kaplan–Meier method and significance assessed by the log-rank test ($P<0.05$).

Results

Graft characteristics

The dose of CD34+ cells given was significantly higher in patients who received CD34+ blood cells purified by a CliniMACS device (CliniMACS-purified CD34+ cells, $n=17$) than in those who received unmanipulated PBSC ($n=18$; $P=0.0027$) or CD34+ blood cells purified by an Isolex system (Isolex-purified CD34+ cells, $n=15$; $P=0.014$; Table 1). The target dose of CD34+ cells (3.0×10^6 CD34 cells/kg) was achieved in 36 donors (72%) by performing two or more leukaphereses. In all, 14 patients (28%) received $<3.0 \times 10^6$ CD34 cells/kg in unmanipulated PBSC ($n=7$), or CliniMACS-purified CD34+ cells ($n=1$) or Isolex-purified CD34+ cells ($n=6$). The dose of CD3+ cells was also significantly higher in those receiving unmanipulated PBSC than in those receiving CliniMACS- or Isolex-purified CD34+ cells ($P=0.0004$ or 0.0009, respectively). As the Isolex systems have changed over the years, variable CD34+ cell doses from the Isolex system may be related to the use of earlier or later version of this equipment.

Engraftment

Among 39 patients (78%) who survived for ⩾28 days after transplant, 37 (95%) and 25 (64%) achieved neutrophil and platelet engraftment, respectively. There was no significant difference in the number of days required for neutrophil engraftment between patients receiving unmanipulated PBSC (median 14 days, range 10–27 days), CliniMACS- and Isolex-purified CD34+ cells (14, 9–20 and 12, 9–20, respectively). There was also no significant difference in the number of days required for platelet engraftment between patients receiving unmanipulated PBSC (18.5, 0–46 days), and CliniMACS- and Isolex-purified CD34+ cells (14, 9–23 and 16, 12–37, respectively).

A total of 11 patients (22%) died before 28 days (median 12 days range 3–27 days) post transplant without evidence of engraftment. Of these patients, two receiving $<3.0 \times 10^6$ CD34 cells/kg of unmanipulated PBSC or Isolex-purified CD34+ cells died of RRT.

Graft failure and rejection

Graft failure occurred in two patients (5%). One of them had received CliniMACS-purified CD34+ cells from a three-loci-MMRD, mismatched for HLA-A, -B and -DR antigens in the HVG direction, and was given a reduced-intensity conditioning regimen containing ATG and GVHD prophylaxis with prednisolone. This patient developed grade IV pulmonary toxicity and died of pulmonary hemorrhage. The other patient with graft failure received Isolex-purified CD34+ cells from a two-loci-MMRD, mismatched for HLA-B and -DR antigens in the HVG direction, and was given a conventional conditioning regimen containing ATG and GVHD prophylaxis with CYA plus prednisolone. This patient developed grade III stomatitis and gastrointenstinal toxicity and died of TMA after secondary allogeneic BMT. Additionally, they received purified CD34+ cells mismatched for HLA-C antigen in the HVG direction.

Three patients (8%) developed graft rejection. All of them had received CliniMACS-purified CD34+ cells and a conventional conditioning regimen including 12 Gy TBI without ATG. One patient received purified CD34+ cells of $<3.0 \times 10^6$ CD34 cells/kg. Their donors were all mismatched for HLA-B and -DR antigens in the HVG direction, of whom two were also mismatched for HLA-C + -A antigens in the HVG direction. They received GVHD prophylaxis with CYA plus methotrexate ($n=2$) or CYA alone ($n=1$).

GVHD

Grades II–IV acute GVHD developed in nine (64%) of 14 evaluable patients receiving unmanipulated PBSC, in three (20%) of 15 receiving CliniMACS-purified CD34+ cells and five (36%) of 14 receiving Isolex-purified CD34+ cells. By stepwise Cox regression analysis, the incidence of grades II–IV acute GVHD in patients receiving purified CD34+ cells (28%) was significantly lower than in those receiving unmanipulated PBSC (64%) (hazard ratio (HR) 0.32; 95% confidence interval (CI) 0.12–0.84; $P=0.022$). While no patients receiving CliniMACS- ($n=4$) or Isolex- ($n=7$) purified CD34+ blood cells with conditioning regimens containing ATG developed grade III or IV acute GVHD, four of 18 patients (22%) receiving CliniMACS- ($n=11$) or Isolex- ($n=7$) purified CD34+ cells without ATG developed grade III or IV acute GVHD. Chronic GVHD developed in three of the 25 evaluable patients (12%; Table 3).

Regimen-related toxicity

Six patients (33%) of those receiving unmanipulated PBSC and 13 (41%) of those receiving purified CD34+ cells developed RRT including grades II–IV organ toxicity before day 100 post transplant according to Bearman's criteria. Additionally, two patients (11%) and five (28%) of those receiving unmanipulated PBSC, and one (3%) and three (9%) of those receiving purified CD34+ cells developed VOD or TMA, respectively (Table 3). Seven of

Table 3 Engraftment, GVHD and regimen-related toxicity

	Manipulation		
	No. (n = 18)	*CD34+ cell selection (n = 32)*	
		CliniMACS (n = 17)	*Isolex (n = 15)*
Median time of engraftment (range) (days)			
Neutrophil	14 (10–27)	14 (9–20)	12 (9–20)
Platelet	18.5 (0–46)	14 (9–23)	16 (12–37)
Graft failure/rejection	0[a]/0	1/3	1/0
Acute GVHD[b]			
0/I	3/2	9/3	5/4
II/III/IV	1/6/2	2/0/1	2/2/1
Median onset (range) (days) of ⩾II acute GVHD	14 (6–77)	26.5 (3–50)	12.5 (5–32)
Chronic GVHD[c] *(onset, days)*			
None/limited/extensive	7/1 (105)/0	9/0/1 (112)	6/1 (101)/0
RRT[d] II/III/IV	2/2/2	1/1/2	2/6/1
VOD/TMA	2/5	1/1	0/2

[a]Number of patients unless indicated otherwise.
[b]A total of 43 patients who developed acute GVHD within 28 days or who survived ⩾28 days after transplant were evaluated for acute GVHD.
[c]A total of 25 patients who engrafted and survived ⩾100 days after transplant were evaluated for chronic GVHD.
[d]Maximum early RRT was graded according to the criteria documented by Bearman *et al.*RRT, regimen-related toxicity; VOD, veno-occlusive disease; TMA, thrombotic microangiopathy; ⩾II acute GVHD, grades II–IV acute GVHD.

11 (64%) patients receiving unmanipulated PBSC and eight of 14 (57%) receiving purified CD34+ cells died of RRT.

Infectious complications

By day 100 post transplant, the most serious treatment-related problem was some form of severe infectious complication, which was seen in 11 patients (61%) receiving unmanipulated PBSC, as follows: bacterial sepsis ($n = 8$, 44%), bacterial pneumonia ($n = 3$, 17%), fungal infection ($n = 2$, 11%) including Candida ($n = 1$) and others ($n = 1$), and adenoviral disease ($n = 2$, 11%). In 20 patients (63%) receiving purified CD34+ cells, these figures were as follows: bacterial sepsis ($n = 12$, 39%), bacterial pneumonia ($n = 2$, 6%), fungal infection ($n = 10$, 31%) including Aspergillus ($n = 2$), Candida ($n = 1$) and others ($n = 7$), and viral disease ($n = 10$, 31%) including CMV ($n = 5$) and adenoviral ($n = 3$) disease, and VZV reactivation ($n = 2$).

Five of these 11 patients (45%) receiving unmanipulated PBSC and eight of these 20 (40%) receiving purified CD34+ cells died of infectious complications before day 100 post transplant. Four of these five patients (80%) receiving unmanipulated PBSC and two of these eight (29%) receiving purified CD34+ cells suffered from infectious complications before achieving neutrophil engraftment. Two patients, who developed grade IV acute GVHD and died of acute GVHD, suffered the complication of Candida infection or CMV disease, respectively, before day 100 post transplant (Table 4).

Eight patients (16%) had carried over active infection to transplant. By day 100 post transplant, three patients who had bacterial infections (methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* or *Stentrophomonas maltophilia*) (infectious sites unknown) before the start of the conditioning regimen suffered sepsis, of whom one died of *P. aeruginosa* sepsis and one of adenoviral pneumonia after recovery from MRSA sepsis. One patient who had pneumonia of unknown origin died of bacterial sepsis, also of unknown origin. Two patients who had Candida liver abscesses or infections of unknown origin and site had *Staphylococcus hemolyticus* or bacterial sepsis of unknown origin, respectively (but recovered). One patient who had active chronic hepatitis C infection died of VOD. One patient who had *Staphylococcus epidermidis* tonsillitis had no infectious complications after transplantation.

Cause of death, relapse, disease progression and survival

In all 11 patients are surviving at a median of 11.7 months (range 4.4–35.5 months) and 39 patients died at a median time of 67 days (range 3–254 days) after transplant. The primary causes of death up to 1 year post transplant in 39 patients (78%) are listed in Table 5. The most common cause of death was treatment-related problem, which was seen in 28 patients (56%). Of the 39 patients who survived ⩾28 day after transplant with confirmed evidence of engraftment, two of 13 (15%) receiving unmanipulated PBSC and two of 26 (8%) receiving purified CD34+ cells relapsed before day 100 post transplant.

Six patients were treated for relapse or disease progression with donor lymphocyte infusion (DLI), the median dose of CD3+ cells being 9.6×10^6/kg (range 1.5×10^5–6.6×10^7/kg), and one underwent a third allogeneic PBSCT. Although grades II acute GVHD developed in two patients who received DLI, and grade III in one, there was no noticeable effect of DLI on disease progression.

Within 168 days after transplant, two of seven patients (29%) undergoing allogeneic PBSCT after prior allogeneic HSCT relapsed and received DLI. One of these two died of

Table 4 Infectious complications before day 100 post-transplant

	Manipulation							
	No. (n = 18)				*CD34+ cell selection (n = 32)*			
	Total	*Neut*	*aGVHD*	*Both*	*Total*	*Neut*	*aGVHD*	*Both*
Bacterial								
Sepsis	8 (2)[a]	6 (2)	1	0	12 (3)	6 (1)	1	0
Pneumonia	3 (1)	3 (1)	0	0	2 (2)	0	0	0
Fungal								
Aspergillus	0	0	0	0	2	1	0	0
Candida	1 (1)	0	1 (1)	0	1	0	0	0
Others[b]	1 (1)	1 (1)	0	0	7 (1)	3 (1)	2	1
Viral								
CMV disease	0	0	0	0	5 (2)	0	1 (1)	0
Adenoviral disease	2	0	1	0	3	0	0	0
VZV reactivation	0	0	0	0	2	1	0	0
CMV antigenemia	6	1	0	3	15	7	0	3

[a]Number of patients (number of deaths) is shown.
[b]Others = *Rhodotorula rubra*, *Pneumocystis carinii* or origin unknown.
Neut, number of patients with incidence of infectious complications before achieving neutrophil engraftment; aGVHD, number of patients with incidence of infectious complications after treating grades II–IV acute GVHD with prednisolone; both, number of patients with incidence of infectious complications before achieving neutrophil engraftment (Neut) and after treating grades II–IV acute GVHD with prednisolone (aGVHD); CMV antigenemia, positive for CMV antigen without diseases caused by CMV infection.

Table 5 Causes of death before 1 year post transplant

	Manipulation	
	No. (n = 18)	*CD34+ cell selection (n = 32)*
Relapse/progressive disease	2 (11%)	9 (28%)
Treatment-related problem	11 (61%)	17 (53%)
Infectious complication	5 (28%)	10 (31%)
Organ toxicity[a]	5 (28%)	5 (16%)
Acute GVHD + infectious complication	1 (5%)	2 (6%)

[a]Organ toxicity = pulmonary hemorrhage (*n* = 2), VOD (*n* = 2), TMA (n = 2), interstitial pneumonia (*n* = 2), intracerebral hemorrhage (*n* = 1) and asphyxia due to oral hematoma (*n* = 1).

relapse and the other died of interstitial pneumonia. However, five of the seven (71%) undergoing allogeneic PBSCT after prior allogeneic HSCT died of infectious complications. The probability of PFS at 1 year in patients undergoing primary allogeneic PBSCT ($n = 33$) or allogeneic PBSCT after prior autologous PBSCT ($n = 10$) was 25.4 and 20.0%, respectively. The probability of OS at 1 year in patients undergoing primary allogeneic PBSCT or allogeneic PBSCT after prior autologous PBSCT was 26.0 and 20.0%, respectively. None of the patients undergoing allogeneic PBSCT after prior allogeneic HSCT survived ⩾196 days after transplant (Figure 1).

There was no significant difference in the incidence of TRM at day 100 post transplant between patients receiving unmanipulated PBSC ($n = 18$, 56%) and purified CD34+ cells ($n = 32$, 44%). The probability of PFS at 1 year in patients receiving unmanipulated PBSC or purified CD34+ cells was 22.2 and 17.6%, respectively ($P = 0.63$). The probability of OS at 1 year in patients receiving unmanipulated PBSC or purified CD34+ cells was 26.7 and 17.9%, respectively ($P = 0.93$; Figure 2).

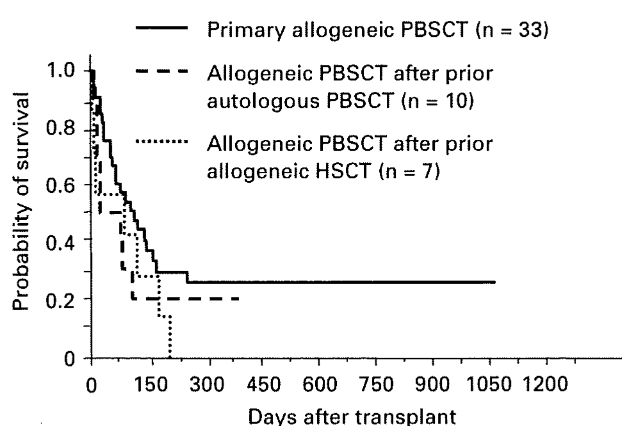

Figure 1 Probability of OS for patients undergoing primary allogeneic PBSCT, and allogeneic PBSCT after prior autologous PBSCT and allogeneic HSCT. The probability of OS at 1 year in patients undergoing primary allogeneic PBSCT or allogeneic PBSCT ($n = 33$, solid line) after prior autologous PBSCT ($n = 10$, dashed line) was 26.0 and 20.0%, respectively. None of the patients undergoing allogeneic PBSCT after prior allogeneic HSCT ($n = 7$, dotted line) survived ⩾196 days after transplant.

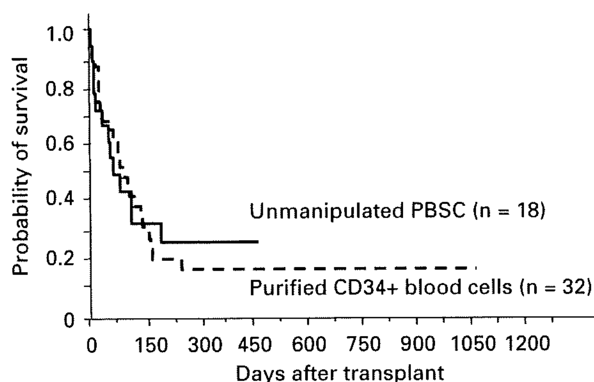

Figure 2 Probability of OS of patients receiving unmanipulated PBSC and purified CD34+ blood cells. The probability of OS at 1 year in patients receiving unmanipulated PBSC ($n=18$, solid line) or purified CD34+ blood cells ($n=32$, dashed line) was 26.7 and 17.9%, respectively ($P=0.93$).

There was no significant difference in the incidence of TRM at day 100 post transplant between patients receiving a conventional ($n=31$, 48%) and a reduced-intensity conditioning regimen ($n=19$, 47%). The probability of PFS at 1 year in patients receiving a conventional or a reduced-intensity conditioning regimen was 26.3 and 16.2%, respectively ($P=0.86$). The probability of OS at 1 year in patients receiving a conventional or a reduced-intensity conditioning regimen was 26.3 and 17.0%, respectively ($P=0.77$).

Discussion

Our data support the notion that the present approach may be a possible option for a realistic alternative rescue for patients who lack an HLA-matched or a one-locus-mismatched related or unrelated donor, because we found that a significant proportion of patients with high-risk hematologic malignancies survived this procedure. As expected, we found that graft manipulation reduced the incidence of grades II–IV acute GVHD. However, there was no significant difference between the probabilities of PFS and OS at 1 year in patients receiving unmanipulated PBSC and purified CD34+ cells. We confirmed that transplant-related problems, particularly infectious complications, before day 100 post transplant were still major obstacles to the success of this therapy.

In the present analysis, we found that there were no significant differences in the time interval to neutrophil engraftment between patients receiving unmanipulated PBSC and purified CD34+ cells. On the other hand, we found that all graft failures (5%, 2/39) and rejections (8%, 3/37) were observed in those receiving purified CD34+ cells. We previously reported that in 13 Japanese children receiving purified CD34+ cells from a partially MMRD,[28] the observed rates of graft failure and rejection were 31% (4/13) and 22% (2/9), respectively. Kato *et al*[29] analyzed the clinical course in 135 young Japanese patients who underwent allogeneic HSCT with purified CD34+ cells from a haploidentical related donor, and found that graft failure occurred in 13% (13/103) of the patients with malignant diseases. Hence, the incidence of graft failure in the current study appears to be less than those in the previous Japanese reports. However, Aversa *et al*[30] reported that graft failure and rejection was 5% (2/43) and 0% (0/41), respectively. Recently, Redei *et al*[31] showed that all evaluable patients, who received $\geqslant 5\times10^6$ CD34+ blood cells purified by an Isolex system without cryopreservation, and were given a conditioning regimen including high-dose TBI, thiotepa, fludarabine and ATG without post transplant immunosuppressive treatment, established successful neutrophil engraftment. As we felt we were able to employ less immunosuppressive conditioning regimens to reduce transplant-related problems, we assumed that graft failure and rejection occurred at higher rates in our study than these two published studies. Additionally, in an attempt to reduce the risk of graft failure, various methods, including purification of blood cells to acquire very large numbers of CD34+ cells, follow-up DLI or T-cell add-back, and various types of anti-lymphocyte or thymocyte antibodies have been investigated,[27,32,33] but we could not find a benefit of employment of these well-established methods in our data. The explanation may again be that most patients were heavily pretreated prior to allogeneic PBSCT from two- or three-loci-MMRD . On the other hand, because all patients with graft failure and two of three of those with graft rejection had received purified CD34+ cells mismatched for HLA-C antigen in the HVG direction, matching for this locus may be an important factor to prevent graft failure and rejection when using purified CD34+ cells. The results of our study again highlight the notion that this approach with purified CD34+ cells should be conducted in a well-designed larger study, focusing on the prevention of graft failure and rejection.

As expected, in this study, the incidence of grades II–IV acute GVHD was significantly lower in patients receiving purified CD34+ cells (28%) compared with unmanipulated PBSC (64%). Similarly, several other studies reported that graft manipulation reduced the incidence of grades II–IV acute GVHD (range 0–21%).[29–31] As our patients received higher doses of CD3+ cells contained in the Isolex-purified CD34+ cells, or less immunosuppressive conditioning regimens with or without various kinds of GVHD prophylaxis, the incidence of grades II–IV acute GVHD in patients receiving purified CD34+ cells may be higher in our study than in other studies. Nonetheless, overall, PFS and OS did not differ significantly between patients receiving unmanipulated PBSC and purified CD34+ cells. This may have been due to the many transplant-related problems before day 100 post transplant observed with this approach. Ruggeri *et al*[34] showed that donor-versus-recipient natural killer (NK)-cell alloreactivity could protect patients against GVHD in allogeneic haploidentical HSCT. Procedures for purifying CD34+ cells have recently been introduced for allogeneic PBSCT from two- or three-loci-MMRD with the development of the CliniMACS and Isolex systems. Although further studies are needed to determine whether cell separation approaches can be applied to this approach, our data suggest that technological advances in cell separation of

PBSC allografts may influence the incidence of acute GVHD.

We could not find risk factors of RRT, VOD and TMA in pretransplant clinical status, such as PS and prior HSCT, in pretransplant treatment, such as conditioning regimens, or in post transplant treatment, such as GVHD prophylaxis and G-CSF use. Recently, new methods of transplantation, that is, nonmyeloablative pretransplant conditioning with or without ATG,[35] and improvements in supportive care in allogeneic HSCT, such as prevention and treatment of GVHD and infectious complications, have been introduced. However, none of the patients who underwent prior allogeneic HSCT survived ⩾196 days after transplantation. Five of the seven (71%) undergoing allogeneic PBSCT after prior allogeneic HSCT died of infectious complications. These results emphasize that this approach after prior allogeneic HSCT should be conducted in a well-designed study, focusing on the prevention of infectious complications, regardless of the application of conventional or reduced-intensity conditioning regimens.

We found that the major cause of death was infectious complications before 1 year post transplant (30%, 15/50), as has also been reported in several other studies (range 33–83%).[7,28–31] In our study, 20 patients (40%) suffered from bacterial sepsis before day 100 post transplant and 12 (24%) suffered from bacterial sepsis before achieving neutrophil engraftment. Thus, the effective prevention and treatment of bacterial infection occurring early after transplantation, especially before achieving neutrophil engraftment, may be of primary importance for the success of this approach. Although Volpi *et al*[36] have shown that G-CSF promotes T-helper (Th)-2 immune deviation, which, unlike Th1 responses, does not protect against fungi, G-CSF after transplant may be useful to reduce the incidence of infectious complications, particularly bacterial ones, because of achieving early neutrophil engraftment. On the other hand, delayed reconstitution of T cells may cause a high incidence of infectious complications, especially fungal and viral infections. Handgretinger *et al*[37] suggested that the transplantation of higher doses of purified CD34+ cells may hasten immune reconstitution. Thus, the number of transplanted CD34+ cells may be important to prevent fungal and viral infections. Although post transplant therapy, such as DLI or T-cell add-back, and reduced GVHD prophylaxis or reduced *in vivo* T-cell depletion may increase the incidence of GVHD, they may reduce the incidence of fungal and viral infections. They should be further investigated in future prospective trials. The incidence of infectious complications was high (75%) in our patients with a carry-over infection before the start of the conditioning regimen. Thus, we should therefore consider selection of patients very carefully and may exclude those with a carry-over infection, particularly MRSA and multidrug-resistant *P. aeruginosa*, from eligibility in any future study.

Our study was limited by the inevitable bias associated with a small population of patients treated at 20 different centers. Nevertheless, we found that a small but significant proportion of patients with high-risk hematologic malignancies survived this approach. In Japan, Kawano *et al*[28] previously reported that the probability of PFS and OS at 1 year was 30 and 40%, respectively, and Kato *et al*[29] reported that the probability of PFS at 67 months was 5.7%. Aversa *et al*[30] reported that the probability of OS at 18 months was 28% (including seven (16%) of 43 standard-risk patients). The probability of OS at 2 years in a study reported by Redei *et al*[31] was 25%. Although this approach is not significantly better in Japan than elsewhere in contrast to allogeneic HSCT from MUD, our data also support the notion that this modality may be a possible option as a realistic alternative rescue for patients who lack an HLA-matched or a one-locus-mismatched related or unrelated donor, regardless of whether they received unmanipulated PBSC or purified CD34+ cells, or the application of conventional or reduced-intensity conditioning regimens.

In conclusion, because only a small number of patients survived this procedure, future studies of allogeneic PBSCT with purified CD34+ cells should be conducted on the prevention of graft failure and rejection, and the observed high risk of treatment-related problems requires eligibility criteria for the selection of patients and the effective prevention and treatment of infectious complications occurring early after transplant. Our data suggested that patients undergoing prior allogeneic HSCT and those with a carry-over infection before the start of the conditioning regimen should also be excluded from this approach because their mortality from infectious complications was extremely high, regardless of whether they received unmanipulated PBSC or purified CD34+ cells, or the application of conventional or reduced-intensity conditioning regimens with or without various kinds of GVHD prophylaxis. The strategy for graft manipulation, and the application of conditioning regimens and GVHD prophylaxis should be further investigated in future prospective trials.

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Appendix

The following transplant centers in Japan participated in this study: Kitakyushu Municipal Hospital, Hamanomachi Hospital, Toyama Prefectural Central Hospital, Harasanshin General Hospital, National Cancer Center Hospital, Osaka University Hospital, Kyushu University Hospital, Institute of Medical Science at the University of Tokyo, Japanese Red Cross Nagoya First Hospital, Kokura Memorial Hospital, Chiba University Hospital, Kyoto Prefectural University of Medicine Hospital, Meitetsu Hospital, Toranomon Hospital, Kashiwa Hospital at Jikei University, Kyoto First Red Cross Hospital, Osaka City University Hospital, Tokai University Hospital, Kagawa Medical University Hospital, Beppu National Hospital, Kameda General Hospital, University of Tokyo Hospital, Kansai University of Medicine Hospital, Osaka National Hospital, Social Insurance Kyoto Hospital, Matsushita Memorial Hospital, Osaka Red Cross Hospital, Nagoya Daini Red Cross Hospital, Kanazawa University Hospital, Kurobe City Hospital, Kumamoto National Hospital and Ryukyu University Hospital.

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Cidofovir for treating adenoviral hemorrhagic cystitis in hematopoietic stem cell transplant recipients

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Summary:

Adenovirus (AdV) infection is an important cause of morbidity and mortality in hematopoietic stem cell transplant (HSCT) recipients. We treated 16 patients with AdV hemorrhagic cystitis (HC) following HSCT with cidofovir (CDV; 1 mg/kg/day, three times weekly for 3 weeks). Patients included 10 males and six females with a median age of 50 years (range 10–62). Two of the 16 patients were unevaluable because of early death from nonadenoviral causes. CDV therapy cleared AdV from urine in 12 of 14 patients (86%). Of 14 patients, 10 (71%) showed clinical improvements in HC. Among 14 patients, seven (50%) had avoided renal damage, the most important CDV toxicity. One patient previously treated with foscarnet for cytomegalovirus (CMV) required hemodialysis, and CDV treatment was discontinued. In another patient, CDV treatment was discontinued because of grade 2 nephrotoxicity. Four patients became positive for CMV antigenemia while being treated with CDV, and two developed herpes simplex virus (HSV) stomatitis while being treated with CDV. CDV proved effective in treating AdV HC in transplant patients. However, CDV at 1 mg/kg/day given three times weekly failed to prevent breakthrough infection with CMV and HSV in some patients.
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Adenovirus (AdV) infections including hemorrhagic cystitis (HC)[1–3] are emerging as life-threatening complications in hematopoietic stem cell transplant patients.[4] Reported occurrence rates of AdV infection complicating allogeneic hematopoietic stem cell transplantation (HSCT) vary from 5 to 21%,[1,5–8] and reported mortality rates have ranged from 7.7 to 38%.[6,9–11]

For treatment of AdV infection, reduction of immunosuppression[8] or infusion of donor lymphocytes[12] have been proposed. However, since AdV infections often occur in the presence of severe graft-versus-host disease (GVHD), immunotherapy may not be feasible. While specific anti-AdV therapy is therefore needed, no presently available drug has been proven to be effective, although some treatment success with ganciclovir (GCV),[13] vidarabine (AraA)[14] and ribavirin[15,16] have been reported. Unfortunately, these results could not be reproduced.[17] Cidofovir (CDV), a monophosphate nucleotide analogue of cytosine that inhibits viral DNA polymerase, demonstrates *in vitro* and *in vivo* activity against several viruses including herpesviruses, AdV, papilloma viruses, polyoma viruses, and poxvirus.[18] Several reports have described the effectiveness of CDV in post-transplant AdV disease.[19,20] The dose-limiting toxicity of intravenous CDV, when given at the recommended dose of 5 mg/kg once weekly, is nephrotoxicity.[18] Recently, a smaller, more frequent dose of CDV, 1 mg/kg/day three times weekly, demonstrated efficacy for treatment of post-transplant AdV infection.[20] In the present study, we have prospectively evaluated both toxicity and efficacy of CDV treatment for AdV HC in transplant patients. In all, 16 transplant patients were treated with CDV at a dose of 1 mg/kg, three times weekly for 3 weeks.

Materials and methods

Diagnosis of AdV HC

To exclude regimen-related HC, only patients who developed macroscopic hematuria with clinical signs of cystitis newly appearing *de novo* at least 10 days after HSCT and also had no tendency toward generalized bleeding or bacteriuria were considered to have HC.[21] According to previously reported criteria with minor modifications,[22] the severity of HC was graded as mild, sustained microscopic

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hematuria; moderate, gross hematuria and dysuria without clots; severe, gross hematuria and dysuria with clots. At the onset of HC, a urine specimen was obtained for viral culture and polymerase chain reaction (PCR). For rapid diagnosis, immunochromatography was performed (Adenocheck; Santen, Osaka, Japan). All patients underwent all the three diagnostic modalities (viral culture, PCR, and immunochromatography). When AdV was detected by one or more of these methods, a diagnosis of AdV HC was made.

Viral culture from urine

A 2 ml volume of urine was centrifuged overnight at 20 000 *g* and the sediment was added to culture of Hep-2 cells for up to 4 weeks. When a cytopathic effect of viral infection was observed, viral species were identified using monoclonal antibodies against AdV. Viral culture was carried out before the initiation of CDV treatment and 1 week after the last dose of CDV. The clearance of AdV was defined as the negative viral culture after treatment.

PCR of urine samples

After 2 ml of a urine sample was centrifuged at 15 000 *g* for 1 h at 4°C, the sediment was resuspended in 100 μl of PBS. DNA was purified using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Next, 5 μl of purified DNA was subjected to PCR assay using a GeneAmp Kit and a GeneAmp PCR System 9600 (Perkin-Elmer, Boston, MA, USA). Primers used to screen for AdV infection were AD185S (5′-tccagcaacttcatgtccatgg-3′) and AD 185A (5′-tcgatgacgccgcggt-3). The size of the final products was confirmed by 3% agarose gel electrophoresis.[21]

Patient characteristics

In total, 16 patients were treated with CDV (10 males and six females with a median age of 50 years, ranging from 10 to 62). All patients had AdV HC. In all, 14 patients underwent allogeneic HSCT for acute myelogenous leukemia (AML) ($n = 1$), acute lymphoblastic leukemia (ALL) ($n = 2$), adult T-cell leukemia/lymphoma (ATL) ($n = 2$), chronic myelogenous leukemia ($n = 1$), myelodysplastic syndrome (MDS) ($n = 1$), multiple myeloma (MM) ($n = 2$), malignant lymphoma (ML) ($n = 1$), and severe aplastic anemia (SAA) ($n = 3$). Two other patients with systemic sclerosis underwent CD34+ cell autologous HSCT. Among allogeneic transplants, sources of stem cells were as follows: three from HLA-identical family donors, one from a DR-mismatch family donor, two from haplo-identical family donors, five from unrelated donors, and three from unrelated cord bloods. Patients No. 3 and no. 12 received antithymocyte globulin (ATG) as part of conditioning (Table 1).

Patient No. 2 died of cerebral infarction 5 days after initiation of CDV treatment, while patient No. 13 died from fungal pneumonia 11 days after initiation of CDV treatment. As these two patients with early death unrelated to AdV were excluded from analysis, 14 patients were evaluable. All patients received immunosuppressive therapy including cyclosporine, tacrolimus, and a steroid, as shown in Table 2. Serotypes of AdV isolated from urine were type 11 (11 patients), type 35 (one patient), or not determined (two patients). Onset of AdV HC ranged from 17 to 142 days after post transplantation (median, 37). Intervals between the onset of AdV HC and CDV treatment ranged from 0 to 56 days (median, 3). Two patients received AraA for treatment of AdV HC (Table 2).

CDV treatment

All of the patients gave their written informed consent in accordance with the requirements of the Institutional Review Board. The treatment regimen consisted of CDV, 1 mg/kg per day three times weekly for 3 weeks. Oral Probenecid (2 g) was given 3 h before CDV administration,

Table 1 Characteristics of patients

Patient no.	*Sex/age*	*Diagnosis*	*Transplant*	*Stem cell source*	*Use of ATG*	*Recipient CMV Ab*	*Donor CMV Ab*	*Recipient HSV Ab*
1	F/20	ALL/2CR	UBMT	DR mismatch	No	Positive	Negative	Positive
2	F/51	ATL/CR	CBT	B, DR mismatch	No	Positive	NE	Positive
3	M/32	SAA	UBMT	Match	Yes	Positive	Positive	Positive
4	F/54	SSc	autoPBSCT	CD34	No	Positive	NE	Positive
5	M/51	ML	alloPBSCT	Haplo-identical	No	Positive	Positive	Positive
6	F/41	ATL/Ref	alloPBSCT	Identical	No	Positive	Positive	Positive
7	F/52	MM	alloPBSCT	DR mismatch	No	Positive	Positive	Positive
8	M/47	ATL	UBMT	Match	No	Positive	Positive	Positive
9	M/17	SAA	BMT	Haplo	No	Positive	Positive	Positive
10	M/10	ALL/Ref	CBT	Three-loci mismatch	No	Positive	NE	Positive
11	M/36	CML/BC	alloPBSCT	Identical	No	Positive	Positive	Positive
12	M/45	SAA	UBMT	DR mismatch	Yes	Positive	Negative	Positive
13	M/62	MM	UBMT	B, DR mismatch	No	Positive	Negative	Positive
14	M/61	MDS	alloPBSCT	Identical	No	Positive	Positive	NE
15	M/50	AML/Ref	CBT	A, B mismatch	No	Positive	NE	NE
16	F/49	SSc	autoPBSCT	CD34	No	Positive	NE	Positive

ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; ATL = adult T-cell leukemia/lymphoma; SAA = severe aplastic anemia; ML = malignant lymphoma; CML = chronic myelogenous leukemia; CR = complete remission; Ref = refractory; BC = blastic crisis; MM = multiple myeloma; MDS = myelodysplastic syndrome; SSc = systemic sclerosis; BMT = bone marrow transplantation; UBMT = unrelated BMT; CBT = cord blood transplantation; PBSCT = peripheral blood stem cell transplantation; auto = autologous; allo = allogeneic; ATG = anti-thymocyte globulin; CMV = cytomegalovirus; HSV = herpes simplex virus; Ab = antibody; NE = not evaluated.