even among those with unfavorable cytogenetics or those with CK. The detrimental prognostic impact of MK was primarily due to high relapse rates and, importantly, similar results were seen in patients who received allogeneic HCT in CR1. Post-transplant relapse occurred more than 20% more frequently in MK+ AML patients than in those in each of the remaining cytogenetic subgroups. This finding is consistent with published studies.[11,14] Investigators at the University of Minnesota analyzed 134 AML patients, including 17 patients with MK who were allografted in CR1, and showed that the MK classification could significantly predict the risk of post-transplant relapse.[14] A report from the Fred Hutchinson Cancer Research Center described the outcome of 35 patients with MK and 193 patients without MK who underwent allogeneic HCT in CR1, in which the 4-year OS rates were 30 and 65% in those with and without MK.[11] Those results taken together with our present results suggest that allogeneic HCT may be able to improve but not completely override the poor prognosis with MK+ AML. It is widely recognized that allogeneic HCT in CR1 is the treatment of choice for patients with AML at cytogenetically unfavorable risk,[15-17] if they have a suitable donor and are fit enough to undergo the procedure. In this study, allogeneic HCT was given to only 21% of patients with MK+ AML during CR1. This low transplantation rate could partly be due to a short CR1 duration, which likely decreased the chance of receiving allogeneic HCT in CR1. A significantly shorter time to transplantation in our MK+ AML patients might reflect the short duration of their CR1 that precluded an implementation of allogeneic HCT after a relatively long interval after achieving CR. Despite a considerable risk of relapse even after transplantation, it is still conceivable that these cytogenetically very unfavorable patients would benefit from allogeneic HCT. We observed that no patient survived long-term without allogeneic HCT, which is in line with reports from the SWOG study.[9]

Our study has several limitations and the results must, therefore, be interpreted with caution. These limitations include the retrospective nature of the study, and the relatively small number of patients with MK+ AML, especially of those who underwent allogeneic HCT in CR1, leaving room for selection bias or chance effect. However, given that MK+ AML accounted for only 4% of our AML patients in CR, it would be quite impractical to conduct a prospective comparison to assess the role of allogeneic HCT in CR1. Under such conditions, the findings from a large-scale retrospective study could have important implications.

In summary, our data confirm that MK exerts a significantly adverse effect on post-remission outcome in AML patients treated with and without allogeneic HCT in CR1. Although our results suggest that allogeneic HCT is already an available treatment of choice, the development of alternative therapies is warranted for this patient population.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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IMAGES IN HEMATOLOGY

Ocular palsy associated with aggressive NK-cell leukemia

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A 22-year-old woman was referred to our hospital with a 1-month history of recurrent fever, subacute onset of bilateral orbital pain, and diplopia. Physical examination revealed the limits of supraduction of her right eye and infraduction of her left eye. A bone marrow aspirate from the ilium showed scattered large immature-looking lymphocytes (15.8%) with pale cytoplasm, fine nuclear chromatin, and nucleoli (Fig. 1). These cells were stained positively with CD56, TIA-1, granzyme, and perforin, and were stained negatively with CD3. In situ hybridization for EBER demonstrated that the cells were EBV-positive. A diagnosis of aggressive NK-cell leukemia was made.

Magnetic resonance imaging of orbital cavity showed marked enlargement, and diffuse high intensity signals in the right superior rectus muscle and left inferior rectus muscle on fat-saturated T2-weighted image (Fig. 1). These lesions were diffusely contrast-enhanced on fat-saturated T1-weighted image (Fig. 2).

After two courses of dexamethasone, methotrexate, ifosfamide, L-asparaginase, and etoposide chemotherapy, she underwent cord blood transplantation with a preparative regimen comprised of etoposide, cyclophosphamide, and total body irradiation. She achieved complete remission, and her ocular manifestations completely resolved.

We could not perform biopsy of the lesions due to the poor general condition of the patient, and the exact etiology

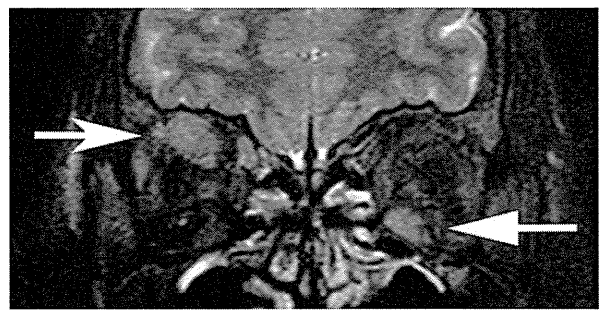

Fig. 1 MRI of orbital cavity showed the marked enlargement, and diffuse high intensity signal in the right superior rectus muscle and left inferior rectus muscle on fat-saturated T2-weighted image

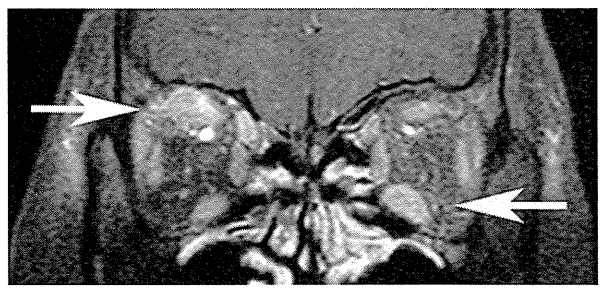

Fig. 2 Eye lesions were diffusely contrast-enhanced on fat-saturated T1-weighted image

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of her ocular manifestation remains to be elucidated. While her ocular symptoms occurred in parallel to the progression of the leukemia, a number of possibilities for the cause of her ocular involvement can be raised, including tumor infiltration, infection, or paraneoplastic myositis. An accumulation of cases of ocular involvement associated with NK-cell leukemia is required.

Conflict of interest None declared.

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ORIGINAL ARTICLE

A randomized controlled trial of plasma real-time PCR and antigenemia assay for monitoring CMV infection after unrelated BMT

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Preemptive therapy is the standard strategy for preventing CMV disease after allogeneic hematopoietic SCT. In this study, unrelated BMT recipients were randomly assigned to a plasma real-time PCR group or an antigenemia group to compare the value of these monitoring tools for CMV reactivation. Ganciclovir (GCV) was started at 5 mg/kg/day when PCR reached 300 copies per ml or when antigenemia reached three positive cells per two slides. A total of 88 patients were randomized into the antigenemia group ($n=45$) or the PCR group ($n=43$). A significantly higher number of patients reached the threshold in the antigenemia group than in the PCR group (73.3 vs 44.2%, $P=0.0089$). However, only three patients (one in the antigenemia group and two in the PCR group) developed early CMV disease. These patients exclusively had colitis and were successfully treated with GCV or foscarnet. The median number of antigenemia-positive cells at the start of GCV was 47 in the PCR group. These findings suggest that antigenemia assay with the current cutoff was too sensitive and led to unnecessary use of GCV. However, the appropriateness of the threshold may be different by the methodology used, and therefore, it is difficult to generalize.

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Introduction

Cytomegalovirus infection is a frequent complication after allogeneic hematopoietic SCT. Universal prophylaxis with ganciclovir (GCV) did not improve the transplantation outcome because of neutropenia caused by GCV.[1,2] Therefore, the initiation of GCV triggered by the detection of CMV reactivation is currently the standard strategy for preventing CMV disease.[3–5] A CMV antigenemia assay has been widely used to monitor CMV reactivation. However, the details of preemptive therapy still need to be clarified, including the threshold number of antigenemia-positive cells for deciding when to start GCV, the dose and duration of GCV and so on. We previously showed that a risk-adapted preemptive therapy, in which the cutoff number of antigenemia-positive cells for deciding when to start GCV was changed according to the risk for CMV disease, was appropriate in allogeneic SCT recipients, but the incidence of neutropenia was still high.[6] Therefore, in the next study, we evaluated the feasibility of preemptive therapy with low-dose GCV, and the findings showed that the initial dose of GCV could be safely decreased to 5 mg/kg.[7]

The PCR used to detect CMV DNA has also been investigated for its ability to monitor CMV reactivation.[8] PCR using whole blood samples might be too sensitive as a trigger for deciding when to start preemptive therapy compared with an antigenemia assay or PCR using plasma samples.[9,10] However, the recent development of real-time PCR has enabled the quantification of CMV DNA. Several studies have shown the feasibility of preemptive therapy guided by real-time PCR monitoring using either whole blood or plasma samples.[11–14] As for whole blood real-time PCR, Gerna *et al.* performed two randomized controlled trials of PCR and antigenemia, one in young patients (0–25 years old) and the other in older patients (20–67 years old).[12,13] They showed that a threshold value of 10 000

copies per ml for determining when to start GCV by whole blood PCR significantly reduced the use of GCV compared with a threshold in which GCV is started at any level of positive antigenemia. However, the study included heterogeneous patients in terms of donor type, stem cell source and GVHD prophylaxis. In particular, antithymocyte globulin was used in approximately half of the patients, and this may have strongly affected the incidence of CMV reactivation and disease.[15,16] In addition, preemptive therapy guided by antigenemia assay could be more appropriately performed by using a cutoff based on the number of positive cells.

Therefore, we performed a randomized controlled trial of plasma real-time PCR with a cutoff of 300 copies per ml and an antigenemia assay with a cutoff of three positive cells per two slides in a homogenous population of unrelated BMT recipients who received GVHD prophylaxis with a calcineurin inhibitor and MTX.

Patients and methods

Patients

Patients were eligible for the study if they were between 20 and 55 years old, would undergo BMT without *in vivo* or *ex vivo* T-cell depletion from an HLA-matched unrelated donor using a myeloablative conditioning regimen and had a good performance status without significant organ dysfunction, as defined in the protocol. Either the donor, the recipient or both must have been seropositive for CMV. Prophylaxis against GVHD was limited to a combination of CYA and MTX, but a combination of tacrolimus and MTX was allowed after June 2002. Patients were enrolled before starting a conditioning regimen, but randomization was performed between day 10 and day 12 after transplantation to exclude patients who developed significant organ dysfunction early after transplantation. This study was approved by the institutional review board of each participating center and a written informed consent was obtained from each patient (UMIN-CTR C000000347).

CMV monitoring methods

Cytomegalovirus antigenemia assay was performed as described previously.[17] In brief, 1.5×10^5 peripheral blood leukocytes were attached to a slide using a cytocentrifuge and fixed with formaldehyde. The cells were sequentially immunostained with MoAb C10/11 (Clonab CMV; Biotest, Dreieich, Germany) and reacted with goat alkaline phosphatase-labeled anti-mouse Ig (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan). Under a light microscopy, CMV-positive cells were counted and the results are presented as the sum of the number of positive cells per two slides.

Real-time PCR was performed using primers and a TaqMan probe for immediate early genes using serum samples.[18] Briefly, DNA extracted from 100 μl of plasma was subjected to PCR using TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA) and the PCR product was detected as an increase in the fluorescent intensity using ABI Prism 7700 (PE Biosystems). Real-time fluorescent measurements were taken and a threshold cycle (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded 10 times the baseline fluorescence. A standard curve was constructed using the CT values obtained from serially diluted DNA extracted from a plasmid that contains the respective region of CMV. The CT values from the clinical samples were plotted on the standard curve and the copy number was calculated automatically using Sequence Detection System version 1.6 (PE Biosystems).

Preemptive therapy against CMV disease

Patients were randomly assigned to the antigenemia group or the PCR group using a random block design. Assignment was stratified by the institute, age and the presence or absence of GVHD at the time of randomization. CMV reactivation was monitored weekly by both the antigenemia assay and PCR in all patients, but only the results of the assigned monitoring method were returned to the physicians. Preemptive therapy with GCV was started at an induction dose of 5 mg/kg/day when three or more CMV-positive cells per two slides were detected in the antigenemia group and 300 or more CMV DNA copies per ml were detected in the PCR group. The dose of GCV was increased to 10 mg/kg/day when a rising CMV load was observed. The dose of GCV was decreased to 5 mg/kg/day when a declining CMV load was observed in patients who were receiving GCV at 10 mg/kg/day. A rising and declining CMV load was defined as an increase and decrease in the CMV load by 50% or more of the previous value, respectively. However, changes in antigenemia-positive cells by less than five cells per two slides and changes in the DNA copy number by less than 500 copies per ml were regarded as a stable CMV load. When the CMV load fell below the threshold to start GCV, the dose of GCV was decreased to 5 mg/kg/day, if the patient was receiving GCV at 10 mg/kg/day, and GCV was discontinued if the patient was receiving GCV at 5 mg/kg/day. The dose of GCV was adjusted according to the renal function.[19] CMV monitoring was continued until all of the following three requirements were fulfilled: (i) More than 100 days had passed after transplantation; (ii) More than 2 weeks had passed after the last administration of GCV; and (iii) Absence of the use of (methyl-)prednisolone at 0.5 mg/kg/day or more.[20]

Definition of CMV disease

All patients with symptoms compatible with CMV disease such as interstitial pneumonia, colitis and gastritis underwent extensive pathological and microbiological examination of biopsy specimens. The diagnosis of CMV disease was made by histopathological examination and immunochemical staining of biopsy specimens. However, CMV retinitis was diagnosed when CMV DNA was detected by PCR using aqueous humor samples associated with characteristic retinal changes by ophthalmoscopy. Early and late CMV diseases were defined as those occurring before and after day 100, respectively.

Statistical considerations

The primary end point of the study was the incidence of early CMV disease. We defined success as the absence of CMV disease before day 100. Noninferiority was predefined as a difference in the success rates between the antigenemia group and the PCR group of no more than 10 percentage points. On the basis of the assumption of a success rate of 95% in the PCR group and 90% in the antigenemia group, 39 patients in each treatment group were required to show noninferiority with an alpha error of 5% and a power of 80%, which permitted a 10% difference in the success rate. On the basis of the assumption of a 20% loss of patients between the enrollment and randomization, a total of 96 patients needed to be enrolled in this study. Comparisons for dichotomous and continuous variables between groups were performed with Fisher's exact test and *t*-test, respectively. Pearson's correlation coefficient was calculated to compare the results of the two monitoring methods after logarithmic transformation.

Results

Incidence of CMV reactivation and the use of GCV

A total of 96 patients were enrolled in the study between January 2002 and March 2007. Among these patients, eight patients were excluded because of the use of tacrolimus as GVHD prophylaxis in one, negative CMV Ab in both the donor and recipient in one and organ dysfunction after the conditioning regimen in six. Therefore, a total of 88 patients were randomized into the antigenemia group ($n=45$) or the PCR group ($n=43$) (Figure 1). There were no differences in age, sex, background disease, CMV serostatus, conditioning regimen or GVHD prophylaxis between the two groups (Table 1). In addition, the incidence of grade II–IV acute GVHD was similar (42 vs 47%, $P=0.67$).

Cytomegalovirus reactivation, defined as a detection of CMV at any level, was more frequently observed in the antigenemia group (40 of 45 patients, 88.9%) than in the

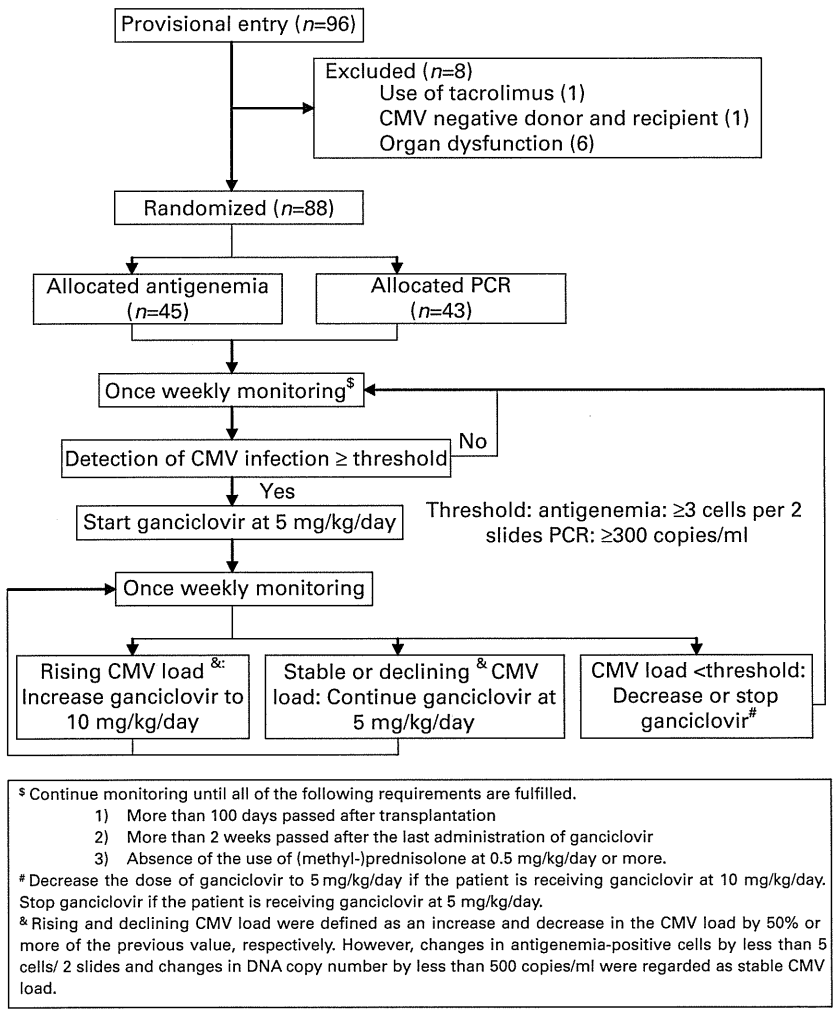

Figure 1 Design of the study.

PCR group (27 of 43 patients, 62.8%) ($P=0.0050$, Table 2). The probability of starting GCV was significantly higher in the antigenemia group than in the PCR group (73.3 vs 44.2%, $P=0.0089$, Figure 2). The results of PCR in the antigenemia group and those of the antigenemia assay in the PCR group were disclosed after the completion of the study. A good correlation was seen between the results of PCR and the antigenemia assay ($P<0.0001$, $r^2=0.38$, Figure 3). Of the 33 patients who received GCV in the antigenemia group, PCR and the antigenemia assay reached the threshold simultaneously in five patients and PCR reached the threshold before starting GCV in only four patients (Figures 4a and 5a). In the other 24 patients, the CMV DNA copy number was persistently below the threshold until GCV was started. On the other hand, in 11 of 19 patients who received GCV in the PCR group, the results of the antigenemia assay reached the threshold earlier in 11 patients and simultaneously in 7 patients (Figures 4b and 5b). The results of the antigenemia assay were persistently below the threshold until GCV was started in only one patient. The median number of antigenemia-positive cells at the start of GCV was 5 (range: 3–102) and 47 (range: 0–2921) in the antigenemia and PCR groups, respectively (Figure 6a, $P=0.0051$). The median CMV DNA copy number was negative (range: 0–4400) and 750 (range: 310–13000) in the antigenemia and PCR groups, respectively (Figure 6b, $P<0.0001$).

Among the 52 patients who received preemptive therapy with GCV at 5 mg/kg/day, only 13 and 7 patients in the antigenemia and PCR groups, respectively, experienced a rising CMV load and required dose-escalation to 10 mg/kg/day, suggesting that the initiation of GCV at 5 mg/kg was appropriate.

Table 1 Patient characteristics

	Antigenemia (n=*45*)	*PCR* (n=*43*)	P-*value*
Pre-transplantation factors			
Median age (range)	41 (20–55)	40 (20–53)	0.82
Sex (male/female)	25/20	24/19	>0.99
HLA mismatch	7 (16%)	9 (21%)	0.59
Background disease			
AML	17	18	
ALL	12	12	
CML	6	3	
MDS	5	7	
Others	5	3	0.57
Donor/recipient CMV status			
Pos./Pos.	28	26	
Pos./Neg.	5	4	
Neg./Pos.	8	6	0.74
Conditioning regimen			
TBI	39	36	
Non-TBI	6	7	0.77
GVHD prophylaxis			
CYA–MTX	25	25	
TAC–MTX	16	16	0.59

Abbreviations: MDS = myelodysplastic syndrome; Neg. = negative; Pos. = positive; TAC = tacrolimus.

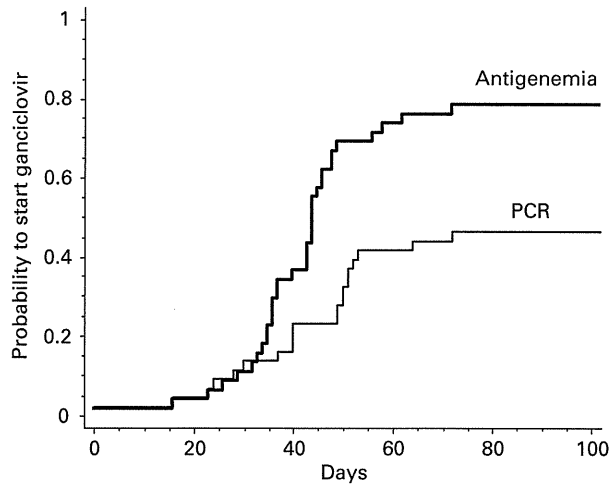

Figure 2 Days to start ganciclovir after transplantation.

Table 2 CMV-related events after engraftment

	Antigenemia (n=*45*)	*PCR* (n=*43*)	P-*value*
CMV reactivation[a]	40	27	0.0050
Start ganciclovir	33	19	0.0089
Duration of ganciclovir (days)	23.2 ± 19.4	20.8 ± 14.2	0.64
Total dose of ganciclovir (mg/kg)	140.8 ± 129.7	118.4 ± 91.2	0.51
Dose escalation to level II	13	7	>0.99
Neutropenia <500 per μl	5	3	>0.99
Stop ganciclovir because of neutropenia	1	0	>0.99
Increase in serum creatinine[b]	8	0	0.039
CMV disease			
Early (before day 100)	1	2	0.61
Late (after day 100)	0	1[c]	0.48

[a]Detection of antigenemia or DNA at any level.
[b]Increase in serum creatinine level by 0.5 mg per 100 ml or more from the baseline level.
[c]The patient developed early CMV disease, which was improved by ganciclovir. However, intestinal symptoms recurred after day 100 and CMV colitis was suspected because of positive antigenemia, although it was not confirmed by biopsy.

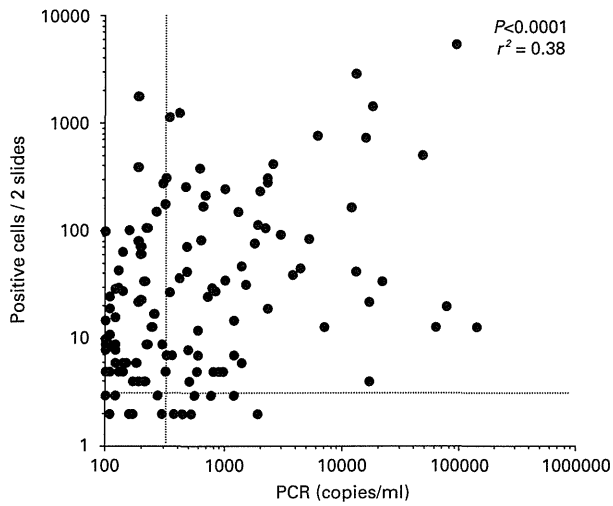

Figure 3 Correlation between the number of positive cells in the antigenemia assay and copy number by PCR.

CMV diseases

Early CMV disease was diagnosed in 1 of the 45 patients (2.2%) in the antigenemia group and 2 of the 43 patients (4.7%) in the PCR group ($P=0.61$). These patients exclusively developed CMV colitis. Another patient in the PCR group showed characteristic retinal changes and was presumptively treated with GCV, although CMV infection was not detected in either the aqueous humor or the peripheral blood. The 95% confidence interval for the difference in the success rate was −10.1 to 5.2%, and thus was just outside the predefined lower limit of −10%. However, as shown in Table 3, the development of CMV disease in the PCR group could not be avoided even if these patients were assigned to the antigenemia group, as either the antigenemia assay and PCR reached the threshold simultaneously (UPN32) or the antigenemia assay did not reach the threshold before the diagnosis of CMV disease (UPN35). All of these patients were successfully treated with GCV or foscarnet, although one patient (UPN35) showed the recurrence of colitis after day 100. None of the other patients developed late CMV disease.

Adverse events during preemptive therapy

The mean duration of preemptive therapy with GCV and the mean total dose of GCV was 23.2 ± 19.4 days and 140.8 ± 129.7 mg/kg in the antigenemia group and 20.8 ± 14.2 days and 118.4 ± 91.2 mg/kg in the PCR group ($P=0.64$ and $P=0.51$), respectively. Neutropenia with a neutrophil count of <500 per μl was observed in 5 of the 33 patients in the antigenemia group and 3 of the 19 patients in the PCR group ($P>0.99$). Only one patient in the antigenemia group required a discontinuation of GCV because of neutropenia. The total dose of GCV was higher in patients who developed neutropenia, but this difference was not statistically significant (163.8 ± 82.5 vs 126.9 ± 121.4, $P=0.42$).

An increase in the serum creatinine level by at least 0.5 mg per 100 ml was observed in 8 of the 33 patients in the antigenemia group and in none of the 19 patients in the PCR group ($P=0.039$). The total dose of GCV was significantly higher in patients who developed renal impairment (255.0 ± 198.0 vs 106.0 ± 45.5, $P=0.0004$).

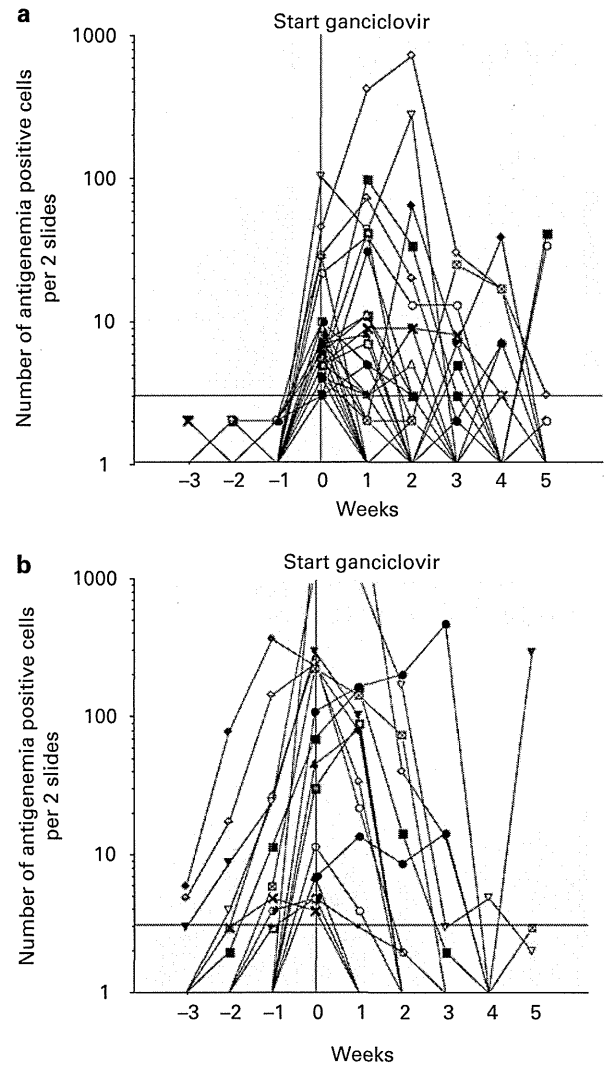

Figure 4 Serial changes in the number of antigenemia-positive cells in patients who received preemptive therapy in the antigenemia group (**a**) and in the PCR group (**b**). Week 0 represents the day ganciclovir was started.

Discussion

In this randomized controlled trial, we compared plasma real-time PCR with a cutoff at 300 copies per ml and an antigenemia assay with a cutoff at three positive cells per two slides as a trigger for deciding when to start preemptive therapy with GCV after unrelated BMT. GCV was used significantly less frequently in the PCR group. A comparison of the number of antigenemia-positive cells and the CMV DNA copy number at the start of GCV treatment clearly revealed that plasma PCR was significantly less sensitive than the antigenemia assay, at least with the current cutoff values. Although the 95% confidence

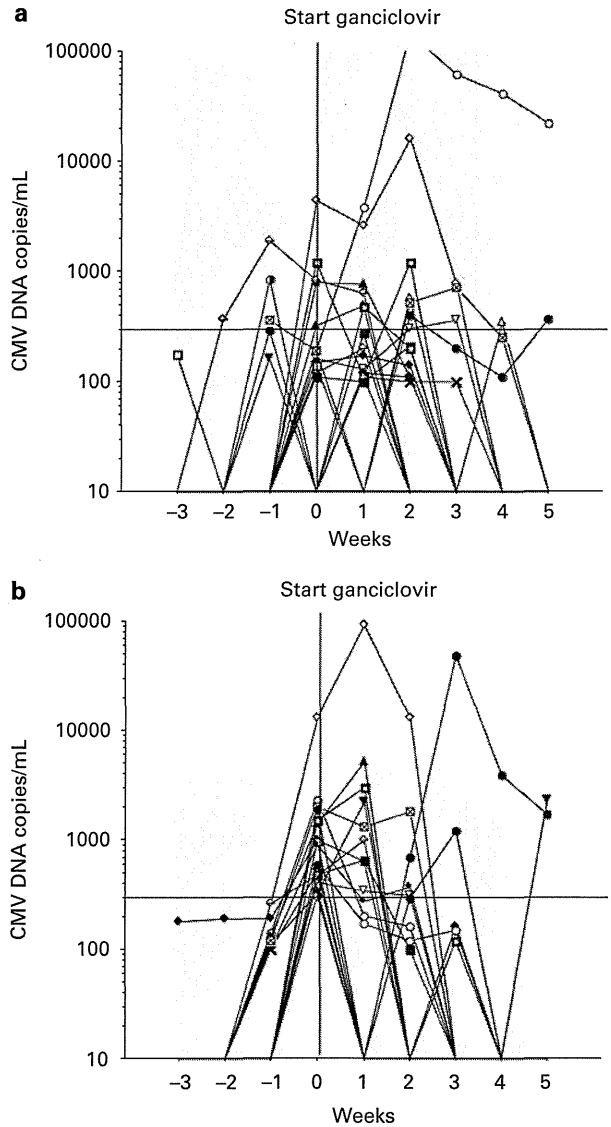

Figure 5 Serial changes in CMV DNA copy number in patients who received preemptive therapy in the antigenemia group (**a**) and in the PCR group (**b**). Week 0 represents the day ganciclovir was started.

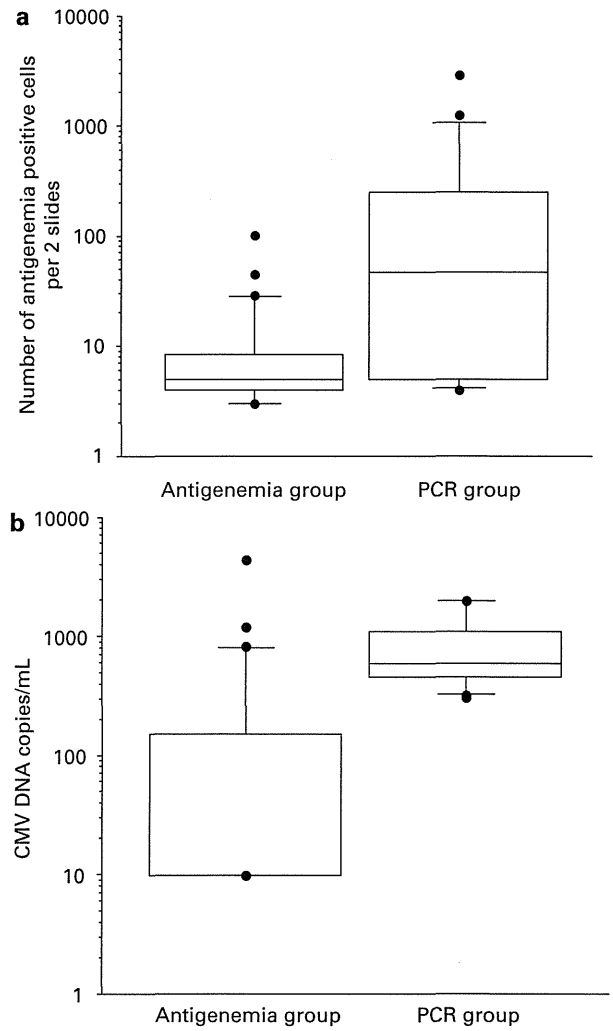

Figure 6 The number of antigenemia-positive cells (**a**) and the CMV DNA copy number at the start of preemptive therapy (**b**), grouped according to the randomization arm. The box-and-whisker plot shows 10, 25, 50, 75 and 90 percentile values. Outliers are indicated by dots.

interval for the difference in the successful prevention rate was just outside the predefined lower limit of −10%, and therefore, we could not show the noninferiority of the PCR group, the incidence of CMV disease was limited to two patients even in the PCR group. In addition, prevention of CMV pneumonia, the main aim of preemptive therapy, was completely achieved in both groups. These findings suggest that an antigenemia assay with a cutoff of three positive cells per two slides was too sensitive and resulted in the unnecessary use of GCV.

The unnecessary use of GCV may be reduced if the cutoff value for the antigenemia assay is increased. The antigenemia assay has already been shown to be not sensitive enough for detecting gastrointestinal involvement by CMV even with a low threshold.[21] In this study, the median number of antigenemia-positive cells at the start of GCV treatment was 47 in the 19 patients who received preemptive therapy in the PCR group. Figure 7 shows the serial changes in the number of antigenemia-positive cells in the patients of the PCR group who developed positive antigenemia that reached the threshold, but who did not receive GCV at that time. In about half of the patients, antigenemia spontaneously became negative without GCV treatment. On the other hand, seven patients developed high-grade antigenemia of over 100 positive cells per two slides. However, GCV was started when the number of positive cells was 260 (median, range: 73–1262 cells) and none of these patients developed CMV disease. Although patients who developed grade II–IV acute GVHD or who received steroid at 0.5 mg/kg or higher experienced high-grade antigenemia more frequently than those who did not

develop grade II–IV acute GVHD and did not receive steroid (Figures 7a and b), the use of GCV was comparable (54.5 vs 40%, $P=0.67$). Thus, although it is difficult to determine the appropriate cutoff value for the antigenemia assay, we thought that it may be worth trying to apply a cutoff value of 20 positive cells per two slides, which we are already safely using in allogeneic hematopoietic SCT from an HLA-matched sibling donor,[20] to transplantation from an unrelated donor.

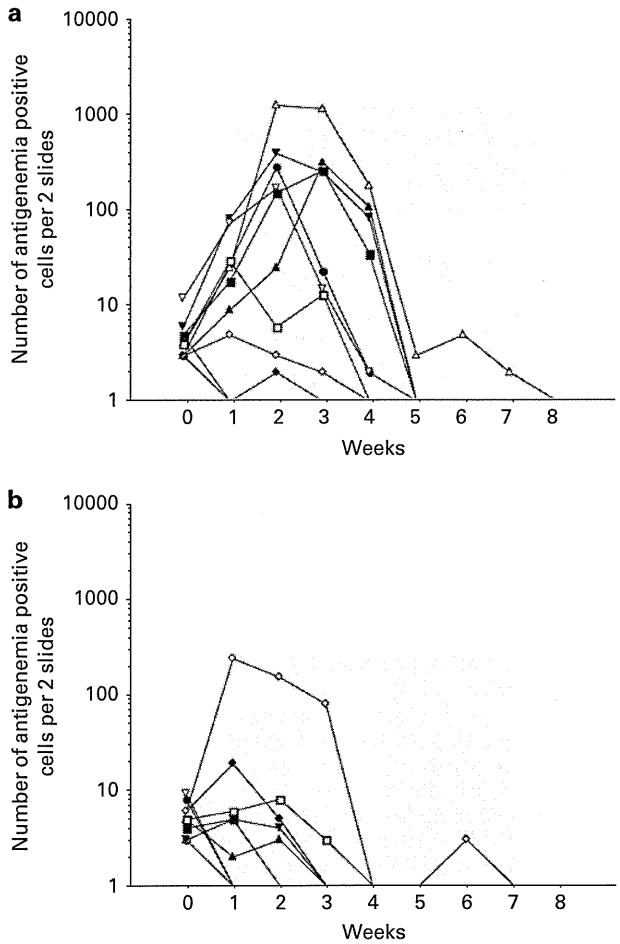

Figure 7 Serial changes in the number of antigenemia-positive cells in the PCR group patients who developed positive antigenemia that reached the threshold, but who did not receive ganciclovir. (**a**) Patients who developed grade II–IV acute GVHD or who received steroid at 0.5 mg/kg or more. (**b**) Patients who did not develop grade II–IV acute GVHD and did not receive steroid.

Although Boeckh *et al.*[3] reported a 14% incidence of early CMV disease using the same cutoff as in the current study, the incidences of positive antigenemia at any level and three or more positive cells per two slides were similar to those in this study (79 and 70% in Boeckh's study and 89 and 73% in the current study). Therefore, the higher incidence of early CMV disease probably resulted from the high incidence (35%) of grade III–IV acute GVHD in their study rather than from the difference in the method used for the antigenemia assay, as acute GVHD is one of the strongest risk factors for CMV disease.

Nevertheless, it is important to note that the sensitivity and specificity of these assays vary depending on the methodology used.[9,22–24] In fact, the unexpected differences in the sensitivities of the two assays in this study could be explained by the difference in the methodology used in the antigenemia assay. The cutoffs used for the antigenemia assay and real-time PCR were determined based on our previous study in which HRP-C7 Ab was used in the antigenemia assay.[18] In this study, however, we used C10/C11 Ab in the antigenemia assay, as this Ab has been used worldwide. Although we did not believe that there are clinical differences between these two antigenemia assays,[6,7,20] we should have tested the correlation between the results of plasma PCR and the antigenemia assay using C10/C11 Ab. Fortunately, the unexpected difference in the sensitivity in these assays contributed to the finding that the antigenemia assay with the current cutoff was too sensitive as a trigger for deciding when to start preemptive therapy. These data are valid only when the same methodology is used, and standardization of the methods is warranted.[25,26]

In conclusion, CMV colitis could not be completely prevented by the current preemptive strategy using the peripheral blood samples, but CMV pneumonia was completely prevented in both groups. The initiation of GCV at 5 mg/kg/day was confirmed to be safe, provided the CMV load continues to be monitored. Plasma PCR with a cutoff at 300 copies per ml seemed to be appropriate for monitoring CMV reactivation after transplantation. The cutoff number of positive cells should be raised above that used here when using an antigenemia assay. However, the appropriateness of the threshold of these assays may be different on the basis of the methodology and patient background, such as the risk of GVHD, and therefore, it is difficult to generalize.

Table 3 CMV load in patients who developed CMV disease

Age/sex	*Acute GVHD*	*Onset/affected organ of CMV disease*		*−3 weeks*	*−2 weeks*	*−1 week*	*Onset*
UPN32 38/M (PCR group)	Grade II	Day 56/colitis	PCR	(−)	260	13 000[a]	93 000
			Ag	(−)	(−)	2921	5467
UPN35 36/M (PCR group)	Grade II	Day 46/colitis	PCR	(−)	(−)	(−)	(−)
			Ag	0	0	2	12
UPN70 38/M (Antigenemia group)	Grade II	Day 50/colitis	PCR	(−)	(−)	110	100
			Ag	2	(−)	5[a]	99

[a]Preemptive therapy was started.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Mycophenolate and Tacrolimus for Graft-Versus-Host Disease Prophylaxis for Elderly After Cord Blood Transplantation: A Matched Pair Comparison With Tacrolimus Alone

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Background. The optimal graft-versus-host disease (GVHD) prophylaxis after umbilical cord blood transplantation has not been established. Our previous observation using single calcineurin inhibitors revealed a high incidence and severity of early immune-mediated complications, especially for older patients or those with poor performance status.
Methods. We conducted a single institute pilot study assessing the safety and effectiveness of mycophenolate mofetil (MMF) and tacrolimus (FK) combination as a GVHD prophylaxis for 29 patients (FK+MMF), and the results were compared with matched-pairs extracted from our historical database who received FK alone as GVHD prophylaxis (control).
Results. FK+MMF group showed superior engraftment rate compared with control group (cumulative incidence until day 60 posttransplant; 90%±0% vs. 69%±1%, $P=0.02$). A cumulative incidence of severe type preengraftment immune reactions was significantly decreased in FK+MMF group (16%±1%) compared with that of control group (52%±2%, $P=0.03$), and, remarkably, there was no nonrelapse mortality (NRM) observed up to day 30 posttransplant in FK+MMF group, whereas 21%±1% of NRM was observed in the control group. However, the incidences of acute and chronic GVHD, estimated overall and progression-free survivals were comparable between two groups.
Conclusions. MMF and FK in combination was well tolerated and decreased early NRM possibly by better control of preengraftment immune reactions. Subsequent NRM or disease progression needs to be overcome to further improve survival.

Keywords: Cord blood transplantation, GVHD prophylaxis, Mycophenolate mofetil, Tacrolimus, Elderly patients.

(*Transplantation* 2011;92: 366–371)

Although umbilical cord blood transplantation (UCBT) has been increasingly used as a curative treatment of hematological diseases, accompanying toxicity, especially early period posttransplant, has been a major problem (*1*, *2*). Our previous observation indicated that elderly patients were more vulnerable to early toxicity posttransplant, with nonrelapse mortality (NRM) being a major cause of treatment failure (*3*). Early immune-mediated complications, termed preengraftment immune reactions (PIR), were significant factors that negatively affected overall survival (OS) (*3–5*).

Various immunosuppressive drugs have been used for graft-versus-host disease (GVHD) prophylaxis in UCBT, including mycophenolate mofetil (MMF), (*6–8*) methotrexate (MTX), (*9–11*) corticosteroids, (*11*) anti-thymocyte globulin, (*12*, *13*), and sirolimus (*14*); mostly in combination with calcineurin inhibitors. So far, no available data indicate that one drug or combination is better than the other.

MMF is an inosine monophosphate dehydrogenase inhibitor that exerts its immunosuppressive effect by blocking the production of guanosine nucleotide synthesis through the

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N.U. performed research design, data analysis, and writing the manuscript; A.W., N.N., K.I., S.T., M.T., H.Y., D.K., N.M., K.M., H.A., Y.A.M., K.I., S.M., and A.Y. participated in the performance of the research; A.W. and S.T. contributed with data analysis and writing of the manuscript; and all authors reviewed the manuscript.

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de novo pathway (*15*). It has been extensively used in solid organ transplantations (*16*) and more recently, in hematopoietic cell transplantation (HCT) (*7, 17–19*). In HCT, less mucosal damage compared with MTX has been observed, (*19–21*) with a comparable incidence of GVHD, suggesting a potential advantage of MMF over MTX. It therefore seemed rational to incorporate MMF in reduced-intensity (RI) UCBT for patients at high risk for NRM. Since December 2005, MMF+tacrolimus (FK) combination was started to be used as GVHD prophylaxis in RI-UCBT as a pilot study for those who agreed to participate. The results were compared with that of those who performed RI-UCBT using FK alone extracted as matched pairs from our historical database.

RESULTS

Patients/Matched Controls

Table 1 shows the demography of the patient characteristics of two groups. A total of 89% of the control patients who had GVHD prophylaxis of FK alone were transplanted from 2004 to 2005, whereas 93% of the patients with FK+MMF were from 2006 to 2007 ($P<.0001$). The differences between the groups did not reach statistical significance in Eastern Cooperative Oncology Group (ECOG) performance status (PS), HCT-specific comorbidity index (HCT-CI) score, history of previous HCT, human leukocyte antigen (HLA) disparity to UCB, and conditioning regimen. The median FK concentrations (11.9±0.33 ng/mL in FK+MMF group vs. 12.6±0.47 ng/mL in control group, $P=0.46$) and the proportions of FK concentration more than or equal to 10 ng/mL during day 0 to the date of engraftment (72.4%±3.1% in FK+MMF group vs. 75.0%±4.0% in control group, $P=0.43$) were comparable in each group.

Engraftment

Twenty-seven patients in FK+MMF group achieved neutrophil engraftment, and all except 1 showed complete donor chimerism. The cumulative incidence of primary engraftment until day 60 posttransplant was 90%±0%, whereas that of control group was 69%±1% ($P=0.02$). Median time to engraftment was 19 days after transplantation both in FK+MMF group (range, 13–32 days) and control group (range, 12–33 days). Among the two patients in FK+MMF group who failed to engraft, one experienced disease recurrence before day 28, and the other experienced rejection of donor cells and was later found to have anti-HLA antibodies against one of the antigens expressed on donor cells. One patient in FK+MMF group who showed mixed chimerism on neutrophil engraftment, when 87.2% of total bone marrow (BM) cells were of donor origin, experienced early BM relapse of leukemia on day 30 posttransplant. There were three patients in control group who experienced hemophagocytic syndrome (HPS) early after transplant and resulted in early death before engraftment, whereas there was no such cases observed in FK+MMF group. Platelet recovery more than 20×10^9/L was observed in 17 patients, with a cumulative incidence of 59%±1% at day 100 posttransplant (median, 40 days; range, 25–70 days), whereas in control group, the cumulative incidence was 52%±1% (median, 40 days; range, 26–62 days, $P=0.69$).

TABLE 1. Patient, treatment, and donor umbilical cord blood characteristics

Characteristic	N (%) of patients FK+MMF	Control	P
Sex			0.38
Male	21 (72)	23 (79)	
Female	8 (28)	6 (21)	
Age (yr)			0.67
Median (range)	62 (52–70)	63 (56–69)	
Age distribution (yr)			
51–55	5 (17)	0	
56–60	4 (14)	9 (31)	
61–65	12 (41)	13 (45)	
66–70	8 (28)	7 (24)	
Diagnosis			0.11
AML/MDS	19 (66)	16 (55)	
ALL	2 (7)	5 (17)	
ML	5 (17)	5 (17)	
CML	0	3 (10)	
AA	3 (10)	0	
ECOG performance status			0.37
0	0	0	
1	22 (76)	17 (59)	
2	5 (17)	9 (31)	
3	2 (7)	3 (10)	
HCT-CI			0.25
0	9 (31)	18 (62)	
1	12 (41)	7 (24)	
2	1 (3)	1 (3)	
≥3	7 (24)	3 (10)	
Disease status			0.78
Standard risk	10 (34)	9 (31)	
High risk	19 (66)	20 (69)	
History of prior HCT			0.16
None	22 (76)	26 (90)	
Autologous	4 (14)	3 (10)	
Allogeneic	3 (10)	0	
Year of transplant			<0.0001
2004	0	11 (38)	
2005	2 (7)	12 (41)	
2006	7 (24)	6 (21)	
2007	20 (69)	4 (14)	
Conditioning regimen[a]			
Flu/Mel 140	8 (28)	1 (3)	
Flu/Mel 80-140/TBI 2-8	13 (45)	25 (86)	
Flu/Mel 80/Tespa 10	0	1 (3)	
Flu/Mel 80-140/Bu 8-16	4 (14)	0	
Flu/Bu 16	0	1 (3)	
Flu/Bu 8-16/TBI 2-4	3 (10)	1 (3)	
Flu/Bu 8/VP-16 450	1 (3)	0	
HLA disparity to UCB			0.22
0 antigen mismatch	1 (3)	1 (3)	
1 antigen mismatch	5 (17)	1 (3)	
2 antigen mismatch	23 (79)	27 (93)	
Total nucleated cell number			0.66
Median ($\times10^7$/kg)	2.4	2.31	
Range ($\times10^7$/kg)	2.0–4.5	1.91–4.76	
$CD34^+$ cell number			0.15
Median ($\times10^5$/kg)	0.9	0.81	
Range ($\times10^5$/kg)	0.11–2.32	0.11–1.9	

[a] Units for each number are as follows: Mel (mg/m^2), TBI (Gy), Tespa (mg/kg), Bu doses: oral (1 dose=1 mg/kg) or iv (1 dose=0.8 mg/kg), and VP-16 (mg/m^2).

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma; CML, chronic myeloid leukemia; AA, aplastic anemia; HCT-CI, hematopoietic cell transplantation-specific comorbidity index; Flu, fludarabine; Mel, melphalan; TBI, total body irradiation; Bu, busulfan; VP-16, etoposide; and UCB, umbilical cord blood; FK, tacrolimus; MMF, mycophenolate mofetil; ECOG, Eastern Cooperative Oncology Group; HLA, human leukocyte antigen.

TABLE 2. Incidence of PIR and GVHD

	FK+MMF (N)	Control (N)
PIR (n=29)		
No. of evaluable[a]	29	28
Yes	22	23
Severe type	4	10
Acute GVHD		
No. of evaluable[b]	27	20
Grade I	4	4
Grade II	7	2
Grade III	7	5
Grade IV	4	3
Chronic GVHD		
No. of evaluable[c]	13	11
Limited	1	2
Extensive	1	2

[a] Those who showed clinical symptoms characteristic to PIR, and those who survived longer than 27 d posttransplant without PIR.

[b] Those who engrafted without disease progression.

[c] Those who survived beyond day 100 posttransplant without disease progression.

PIR, preengraftment immune reactions; GVHD, graft-versus-host disease; FK, tacrolimus; MMF, mycophenolate mofetil.

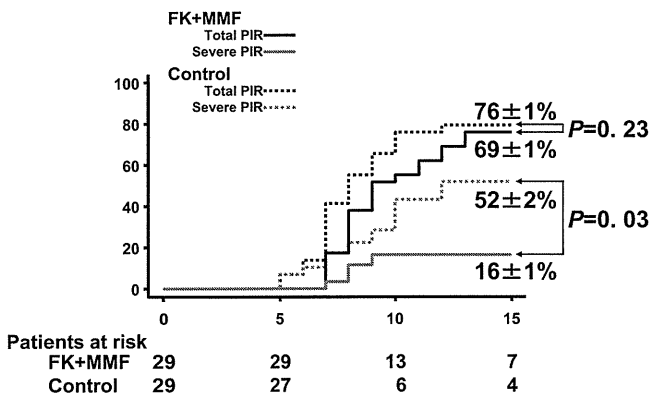

FIGURE 1. Cumulative incidences of preengraftment immune reactions (PIR) after RI-UCBT according to tacrolimus (FK)+mycophenolate mofetil (MMF) or FK alone graft-versus-host disease (GVHD) prophylaxis. The overall incidences of PIR in FK+MMF group (*black solid line*), in control group (*black dotted line*), and the incidences of severe type of PIR in FK+MMF group (*gray solid line*), and in control group (*gray dotted line*) were plotted. There was significant reduction of severe type of PIR in FK+MMF group compared with that in control group (P=0.03).

PIR and GVHD

In FK+MMF group, 22 of 29 patients experienced clinical symptoms defined as PIR, whereas in control group, 23 of 28 evaluable patients did (Table 2). Cumulative incidences of PIR in both groups were comparable each other (76%±1% in control group and 69%±1% in FK+MMF group, P=0.23, Fig. 1) and were similar to that reported in our previous publication (*3*). However, the cumulative incidence of severe type of PIR, defined by the criteria described in materials and methods section, in the FK+MMF group was lower (16%±1%) than that of control group (52%±2%) with statistical significance (P=0.03, Fig. 1).

In FK+MMF group, 22 of 27 evaluable patients developed acute GVHD, and 18 of them were grade II and higher. In control group, 14 of 20 evaluable patients had acute GVHD, and 10 of them were grade II and higher (Table 2). Cumulative incidences of grade II and higher acute GVHD at day 100 posttransplant were 63%±1% in FK+MMF and 35%±1% in control group (P=0.09). Chronic GVHD was observed in two of 13 FK+MMF group and four of 11 control group patients who survived longer than 100 days posttransplant without disease progression (Table 2). Cumulative incidences of chronic GVHD at 2 years posttransplant were 7%±0% in FK+MMF and 16%±1% in control group (P=0.35).

TABLE 3. Causes of death

	FK+MMF, N (%)	Control, N (%)
NRM	9 (45)	11 (65)
GVHD	5 (25)	3 (18)
IPS	4 (20)	1 (6)
Infection	0	5 (29)
CNS complication	0	2 (12)
Relapse/disease progression	11 (55)	6 (35)
Total	20	17

FK, tacrolimus; MMF, mycophenolate mofetil; NRM, nonrelapse mortality; GVHD, graft-versus-host disease; IPS, idiopathic pneumonia syndrome; CNS, central nervous system.

Survival, Disease Progression, and NRM

At the time of analysis, 9 FK+MMF group patients survived for a median of 980 days (range, 145–1430 days) after transplantation, whereas 12 control group patients were alive for a median of 1073 days (range, 49–2071 days). The Kaplan-Meier estimates of OS and progression-free survival (PFS) at 2 year posttransplant in FK+MMF group were 33%±9% and 21%±8%, whereas those in control group were 45%±10% and 34%±9%, respectively. The differences were not statistically significant (P=0.83 for OS, and P=0.75 for PFS).

Thirteen patients in FK+MMF group showed progression of the underlying disease at a median of 84 days (range, 19–344 days) after transplantation, and 11 of these patients died of the disease (Table 3). In control group, 9 patients did so at a median of 126 days (range, 12–1084 days) and 6 died of the disease. The cumulative incidences of disease progression at 2 years were 46%±1% in FK+MMF group and 29%±1% in control group, respectively (P=0.29).

Nine in FK+MMF group died of nonrelapse causes, whereas in control group patients, 11 NRM were observed (Table 3). GVHD and noninfectious pulmonary complications were observed in both groups as cause of death. None of the FK+MMF group died from infections as a sole reason of death, whereas five of the control group did. There was no death before day 30 posttransplant in FK+MMF group, whereas six in control group did. The cumulative incidences of NRM at day 30, 100, 365 were 0%±0%, 21%±1%, 28%±1% in FK+MMF group, and 21%±1%, 35%±1%,

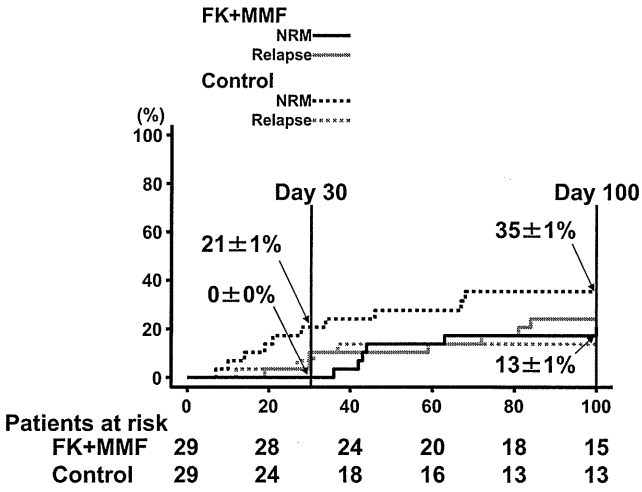

FIGURE 2. Day 100 nonrelapse mortality (NRM) and disease progression. Cumulative incidence estimates of NRM (*black line*) and disease progression (*gray line*) up to day 100 posttransplant for tacrolimus (FK)+mycophenolate mofetil (MMF) group (*solid line*) and control group (*dotted line*) were plotted. There were no NRM within 30 days posttransplant in FK+MMF group, whereas 21%±1% NRM were estimated in control group (P=0.01).

39%±1% in control group, respectively (P=0.01, P=0.17, P=0.29, Fig. 2).

DISCUSSION

The most remarkable observation in this study was that higher rate of neutrophil recovery and no early deaths before day 30 posttransplant were observed in FK+MMF group despite the patients' poor conditions before transplant, that is, all were older than 50 years and 69% of them had some comorbidities. Although the incidence of PIR in FK+MMF group was comparable with control group, the severity of PIR was less and thus did not result in severe organ damage early after transplant. There was no death directly caused by infections in FK+MMF group. We have reported higher incidence of HPS after RI-UCBT, which has been reasoned to be the delayed engraftment or graft failure (*22*). Interestingly, majority of the suffered BM cells were donor cell dominant, indicating HPS was mediated by donor-derived immune cells. Moreover, we have reported HLA mismatch in GVH direction, not in host-versus-graft direction, affected negatively to successful engraftment (*23*). All these facts fit well to the idea that hyperimmune reactions caused by donor cord blood (CB) cells may play crucial role in high rate of early NRM. Because there were no case of HPS in FK+MMF group, MMF may have promoted engraftment by sufficiently suppressing immune reactions of CB cells and preventing development of hemophagocytosis, which may also have reduced the incidence of severe infections. The presence of this type of hyperimmune reactions after UCBT has recently been recognized by others (*24*). The differences in incidence of PIR may have been affected by agents included in pretransplant conditioning, such as antithymocyte globulin, or by GVHD prophylaxis including corticosteroids or intravenous MMF.

Despite the present observation that the combination of MMF and FK succeeded in reducing early NRM, the incidence and severity of GVHD was not altered. Because most of the patients in the present study had advanced disease status, MMF was discontinued or started to be tapered on the day of neutrophil engraftment, which may have been responsible for this results. Much longer administration of MMF has been used in the setting of matched unrelated BM/peripheral blood (PB) transplantation (*7*). In addition, MMF was administered at 15 mg/kg twice daily in this study, which is the common dosing schedule in the settings of solid organ transplant (*16*). Several recent reports from Minnesota and Seattle considered 15 mg/kg three times daily as more appropriate based on pharmacokinetic data obtained from HCT recipients (*7, 17, 25*). A serum concentration measurement of mycophenolic acid, which was not assessed in this study, is needed to determine the optimal dosing of MMF.

Although NRM early after UCBT was significantly reduced in FK+MMF group, OS and PFS at 2 year posttransplant were still comparable with those of control group. Fifty-five percent of the deaths were from disease relapse or progression. Although MMF may have a beneficial effect on early survival after transplant by reducing severe immune reactions, it may increase the risk of disease progression for those who have active disease with a high risk of disease recurrence. According to previous publications, relapse rate is comparable in CB and unrelated BM/PB recipients despite lower incidences of chronic GVHD in CB recipients (*26, 27*), early immune reactions may have impact on reducing disease relapse. Because this is a relatively small sized, retrospective study, the presence of uncontrolled bias cannot be excluded. Prospectively conducted larger studies are warranted to further confirm the results.

In conclusion, MMF, used in combination with FK as GHVD prophylaxis in elderly patients with advanced hematologic diseases with or without comorbidities, may reduce early mortality posttransplant by regulating severe PIR and thus protecting patients from severe organ damage or HPS. An optimal dosing schedule of MMF needs to be determined prospectively using more homogenous populations.

MATERIALS AND METHODS

Patients

The initial pilot study included patients aged 51 years and older who underwent RI-UCBT using MMF+FK combination as GVHD prophylaxis at our institute from December 2005 through December 2007. Patients were eligible for this study if they had any hematologic malignancies at high risk for relapse or severe aplastic anemia refractory to standard immunosuppressive therapy and were unable to find suitable related or unrelated BM/PB donors within reasonable periods relative to their disease conditions. Patients with acute leukemia could be at first remission but at high risk for relapse due to adverse cytogenetic abnormalities, have a previous hematologic disorder, or be at any status beyond first remission. Patients with myelodysplastic syndrome (MDS) had to be refractory anemia (RA) with excess of blasts or chronic myelomonocytic leukemia, or have RA with transfusion dependency or severe neutropenia. Malignant lymphoma (ML) patients had to be beyond first remission. Patients who had end-stage cardiac dysfunction (left ventricular ejection fraction <35%), pulmonary dysfunction (SpO_2 <90% in room air), or active serious infection at the time of transplantation were not eligible. All patients gave written informed consent. Twenty-nine patients were enrolled and subjected to the matched pair analysis as below.

Selection of Matched Controls and Matching Variables

A matched-pair control group (GVHD prophylaxis with FK alone) for 29 patients who used MMF+FK combination was obtained by selecting one of the most recently transplanted control patients from our historical RICBT database from 2004 to 2007 after excluding those who met exclusion criteria of the pilot study described earlier. Controls were individually matched to cases on a 1:1 ratio. Matching was attempted for the following criteria applied in the order listed: age at transplantation (51–60, 61–70 years), disease risk (standard risk vs. high risk, acute leukemia, chronic myeloid leukemia, or ML in complete remission, MDS RA, aplastic anemia patients were categorized as standard risk, and all the others were as high risk), ECOG PS (PS 0–1, 2–3), pretransplant conditioning (busulfan containing vs. others), number of serological HLA mismatch (0–1, 2), HCT-CI (0–1, ≥2), total nucleated cell dose infused (≤2.3, >2.3×10^7/kg), and $CD34^+$ cell dose infused (≤0.8, >0.8×10^5/kg). To avoid any potential selection bias, matching was blinded, and only the patient's initials and pretreatment variables were known. This retrospective analysis was approved by the institutional review board.

One hundred percent matching was achieved for age group; 97% for disease risk (high risk, 66% of FK+MMF patients vs. 69% of control patients; $P=0.78$); 83% for ECOG PS (≥2 score, 32% of FK+MMF patients vs. 41% of FK alone patients; $P=0.16$); 72% for HCT-CI (≥2 score, 28% of FK+MMF patients vs. 14% of control patients; $P=0.19$); and number of serological HLA mismatch (2 antigens, 79% of FK+MMF patients vs. 93% of control patients; $P=0.13$); 86% for pretransplant conditioning (inclusion of busulfan, 24% of FK+MMF patients vs. 7% of control patients; $P=0.07$); 69% for total nucleated cell dose (≤2.3×10^7/kg, 41% of FK+MMF patients vs. 45% of control patients; $P=0.79$); and 62% for $CD34^+$ cell dose (≤0.8×10^5/kg, 45% of FK+MMF patients vs. 48% of control patients; $P=0.79$). Characteristics of the studied patients in both groups were shown in Table 1. Patients' comorbidity was assessed by a previously reported scoring system (*28*).

Donor Selection

UCB units were obtained from the Japanese Cord Blood Bank Network. HLA-A, -B, and -DR antigens were identified by serologic typing. UCB grafts had at least four of six HLA-A, -B, and -DR antigens that were matched to the recipient and had a cryopreserved cell dose of at least 1.9×10^7 nucleated cells per kg of recipient body weight.

Conditioning Regimens and Postgrafting Immunosuppression

Pretransplant conditionings were primarily RI regimens including 125 to 180 mg/m^2 of fludarabine (25 mg/m^2 for 5 days or 30 mg/m^2 for 6 days). Antithymocyte globulin was not incorporated. Granulocyte colony-stimulating factor (G-CSF) was started on day 1 posttransplant. Detailed information is shown in Table 1. Immunosuppressive therapy with FK (0.03 mg/kg continuous infusion, aiming for 12 to 17 ng/mL by at least three times a week measurement) with or without MMF (15 mg/kg twice daily) were started on day −1. MMF was discontinued or started to taper down on the day of neutrophil engraftment in the absence of active GVHD.

Definition of Engraftment, Preengraftment Immune Reactions, and Endpoints

Engraftment was defined as absolute neutrophil count more than 0.5×10^9/L for 3 consecutive days. Chimerism was assessed using fluorescent in situ hybridization in sex-mismatched donor-recipient pairs, or polymerase chain reaction for a variable number of tandem repeats with donor cells detected at a sensitivity of 10% in sex-matched pairs. Whole blood or BM cells were assessed at the time of granulocyte engraftment. Complete donor-type chimerism was defined when donor cells consisted of more than 90% of analyzed cells. PIR was characterized by the presence of at least three of the following symptoms with no direct consequences of infection or adverse effects of medication six or more days before engraftment, as described previously (*4, 5*): a high fever (>38.5°C), skin eruptions, body weight gain greater than 5% of baseline, or peripheral edema. Those who had all four symptoms and at least two of the following criteria indicating severe organ damage were classified as severe type; (1) SpO_2 less than 92% or pleural/pericardial effusions present; (2) serum creatinine level more than or equal to 3 times of baseline; (3) total bilirubin level more than 3 mg/dL or asparatate aminotransferase/alanine aminotransferase levels more than three times of upper limit of normal; and (4) development of hemophagocytosis in BM.

The main parameters analyzed between groups were as follows: (1) cumulative incidences of neutrophil or platelet engraftment; (2) cumulative incidences of NRM and relapse; (3) incidences of PIR, acute and chronic GVHD; and (4) overall and progression-free survival (OS and PFS). The analysis was performed as of April, 2010. OS was calculated from the day of transplantation until death from any cause or last follow-up. PFS was calculated from the day of transplantation until relapse, second transplantation due to engraftment failure, or death from any cause or last follow-up. NRM was defined as death in the absence of disease progression. Deaths occurring after disease progression were categorized as relapse regardless of the cause of death. Infection was considered the cause of death when bacterial, viral, or fungal infection was determined to be the proximate cause of death in patients who had not relapsed. Patients underwent BM aspiration at the time of engraftment or if clinically indicated. Relapse for acute myeloid leukemia, acute lymphoblastic leukemia, MDS, or chronic myeloid leukemia was determined by flow cytometric, morphologic, or cytogenetic evidence of malignant or dysplastic cells with clonal markers similar to those observed before transplantation. Relapse for ML was defined as progressive adenopathy or BM involvement. Acute and chronic GVHD were defined and graded by standard criteria (*29*). Relapse and NRM rates were estimated using cumulative incidence analysis and were considered competing risks (*30*). Similarly, in the analysis of PIR rates, death due to other causes or relapse leading to early withdrawal of immune suppression were considered competing risks.

Statistical Methods

Chi-square test was used to compare patient characteristics between two groups in matched-pair analysis. For continuous variables, Mann-Whitney nonparametric test was used. The probabilities of OS and PFS were estimated and plotted using the Kaplan-Meier method (*31*). Cumulative incidence curves were drawn using Gray's method (*32*). The level of significance in all cases was set at P less than 0.05. The effect of various categoric variables on survival probabilities was studied with the log-rank test. A Cox proportional hazard model with limited variables because of small sample was used to determine the significance of multiple variables in determining these outcomes. All analyses were carried out using StatView statistical software for Kaplan-Meier curve, and S-PLUS software (Mathsoft, Seattle, WA) for cumulative incidence curve.

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LETTER TO THE EDITOR

Unexpectedly high AUC levels in a child who received intravenous busulfan before stem cell transplantation

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A 6-year-old boy underwent a craniotomy with subtotal resection of the cerebellar vermian tumor. Microscopic examination showed medulloblastoma. Brain magnetic resonance imaging after resection did not show residual disease. He subsequently received four cycles of ICE (ifosfamide, cisplatin, and etoposide) chemotherapy followed by whole craniospinal (24 Gy) and local (30 Gy) radiation therapy. He eventually received tandem high-dose chemotherapy. Conditioning regimens for the first and the second were carboplatin (1200 mg/m^2) + thiotepa (750 mg/m^2) and busulfan 1.1 mg/kg/dose intravenous every 6 h for total 16 doses (17.6 mg/kg) + melphalan 70 mg/m^2 once daily intravenous for 2 days (140 mg/m^2), respectively. Autologous PBSC were infused after high-dose chemotherapy. The numbers of CD-34-positive cells were 5.2×10^6/kg in the first and 7.0×10^6/kg in the second, respectively. Engraftment after the second autologous PBSCT was achieved on day 11. Brain magnetic resonance imaging on day 25 of the second autologous PBSCT showed no evidence of recurrence.

On day 64 of the second autologous PBSCT, he developed sudden dyspnea with 90% oxygen saturation in room air. A chest X-ray film showed a ground-glass appearance in both lungs. Chest computed tomography scan also showed bilateral areas of ground-glass opacities, and no centrilobular micronodule. Aspergillus, candida, and cryptococcal antigen for the serum were negative. The β-D-glucan level was significantly elevated (234.4 pg/ml). Intravenous cotrimoxazole therapy was initiated, because pneumocystis pneumonia was thought to have caused pneumonia based on the clinical course. He was placed on mechanical ventilation and methylprednisolone pulse therapy (30 mg/kg × 3 days) was started on day 72. His respiratory condition improved quickly with these treatments and mechanical ventilation was discontinued on day 77. Although polymerase chain reaction for *Pneumocystis jirovecii* in bronchoalveolar lavage fluid was negative, we diagnosed the cause of his pulmonary disorder had been pneumocystis pneumonia. His respiratory condition deteriorated again on day 82. Despite the second course of methylprednisolone pulse and continued cotrimoxazole therapy, he was placed on mechanical ventilation again on day 85 (Figure 1a). No pathogenic bacterial, fungal, or viral agents, including cytomegalovirus were identified by microbiological cultures and serological studies. The β-D-glucan level was also decreased to 33.4 pg/ml. Although various antibiotics, ganciclovir, antifungal drugs, and cotrimoxazole therapy were continued, his pulmonary oxygenation became worse day-by-day. He died on day 133 of the second autologous PBSCT because of respiratory failure (Figure 1b).

An autopsy was performed with the consent of his parents. Microscopically, organizing diffuse alveolar damage was seen, and nuclear enlargement, hyperchromasia, and pleomorphism were seen along alveolar and bronchial epithelium. This cytologic atypia was seen not only in the lung, but also in the urothelium of the renal pelvis. There was no evidence of bacterial, fungal, or viral infection. These pathological findings were consistent with busulfan-induced lung disease. Organizing diffuse alveolar damage is the most common manifestation of busulfan lung toxicity and is associated with bronchiolar and alveolar epithelium atypia.[1] This cytologic atypia is often seen extrapulmonary sites, including urinary bladder, breast, and uterine cervix.[1] The incidence of pulmonary toxicity after high-dose oral busulfan therapy before stem cell transplantation has been reported to be 3.6%.[2] Corticosteroids are effective for treating this disease to various degrees. Although some patients improve, others progress and die.[1]

It has become evident posthumously that busulfan areas under the drug plasma concentration–time curve (AUC) levels at the 1st and 9th doses were significantly elevated (2353 μM × min, 2347 μM × min) (target AUC; 900–1500 μM × min). Busulfan concentrations for the 1st and 9th doses and the accumulation of intravenous busulfan in plasma were assayed using a high-performance liquid chromatography system (Figure 2).[3] Plasma concentrations were analyzed by the non-compartmental method using WinNonlin (version 5.2.1; Pharsight Corp., Mountain View, CA, USA). The AUC from time 0 to infinity (AUC_{inf}) for the 1st dose and at steady state (AUC_{ss}) for the 9th dose was calculated using linear trapezoidal rule. The relationship between high busulfan AUC levels and the occurrence of busulfan-induced lung disease has not been established,[4] although high busulfan AUC levels are commonly associated with hepatic veno-occlusive disease.[5]

The cause of high busulfan AUC levels in our patient is still unclear. He did not have the distinct liver or renal disorder at the time of busulfan administration. Busulfan is metabolized in the liver through conjugation with glutathione by glutathione *S*-transferase (GST) enzymes.[6] GSTA1 is the predominant isoform of GST, which catalyzes the conjugation of busulfan with glutathione. Polymorphisms in GSTA1 are thought to be associated with alterations in the pharmacokinetics of busulfan.[7]

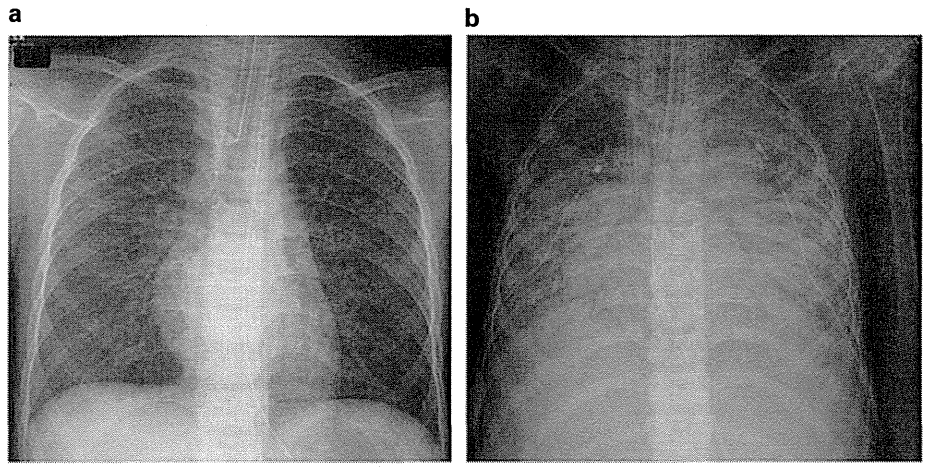

Figure 1 Chest X-ray films. (**a**) (On day 85) bilateral lung fields showed a ground-glass appearance. (**b**) (On day 133) bilateral lung fields showed marked radiopacity and air bronchograms.

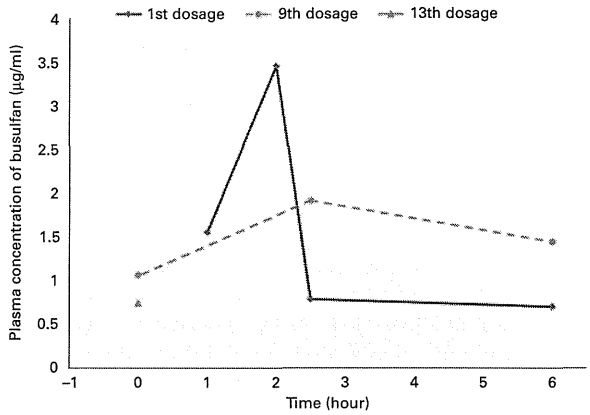

Figure 2 Plasma concentration of busulfan. Peak level of plasma concentration of busulfan is very high (1st dose). Busulfan clearance is poor (before 13th dose).

Johnson *et al.*[7] reported that the GSTA1*B variant had a 2.6-fold higher busulfan AUC level than other variants after intravenous busulfan exposure in the pediatric population. Our patient's genotype of the promoter region of GST A1 by DNA sequencing was GSTA1*A diplotype (-567T, -69C, -52G) which is thought to be more active than the GSTA1 *A/*B. Nevertheless, busulfan AUC levels were significantly elevated. This may indicate that polymorphisms other than GSTA1 polymorphisms may affect busulfan metabolism.

Intravenous busulfan should have a much more predictable pharmacokinetic profile than oral busulfan. Treatment with a fixed dose of 0.80 mg/kg intravenous busulfan achieved the target AUC level (900–1500 μM × min) in 80% of adult patients.[8] The remaining 20% were very close to achieving the target level.[8] A recent European study showed that 91% of children achieved target AUC levels by weight-based dosing.[9] They concluded that this weight-based dosing in children is sufficient without therapeutic drug monitoring and dose adjustment. Our patient received intravenous busulfan according to his body weight, as in the European study. However, his busulfan AUC levels were higher than has been reported elsewhere in the literature.[9,10] To avoid unexpectedly high busulfan AUC levels, therapeutic drug monitoring and dose adjustment should be recommended for all patients who are treated with high-dose busulfan. When therapeutic drug monitoring is not applicable, test dosing of intravenous busulfan before high-dose therapy would be preferable.

Conflict of interest

The authors declare no conflict of interest.

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