Table 2 Outcomes of patients who received RIST for ALL

Patient	*Age/ sex*	*Karyotype*	*Status before RIST*	*Performance status*	*Donor*	*Relapse*	*Reason for DLI*	*Acute GVHD*	*Chronic GVHD*	*Present status*	*Follow-up, days*	*Cause of death*
1	55/F	Unavailable	CR1	1	Identical related	Day+28		II	NE	Dead	85	Leukemia
2	59/F	Others	CR1	1	Identical related	Day+197	Relapse	II	Limited	Dead	299	Severe GVHD following DLI
3	68/F	Normal	CR2	2	Identical related	Day+315	CNS relapse	0	No	Dead	870	Progression after second RIST
4	43/F	t(9:22)(q34;q11)	Primary refractory	3	Mismatched related	No	Prophylaxis	I	Extensive	Death in CR1	406	Bronchitis obliterans
5	56/F	Normal	CR1	0	Identical related	No		0	Extensive	Alive in CR1	1119	
6	50/M	t(9:22)(q34;q11)	Second relapse	0	Identical related	No		II	NE	Death	71	Hemophagocytic syndrome, VOD, GVHD
7	58/M	t(9:22)(q34;q11)	CR2	0	Mismatched related	Day+181		II	Not available	Dead	237	Leukemia
8	31/F	t(9:22)(q34;q11)	Second relapse	0	Identical related	Day+36	Relapse	0	NE	Death	134	Second RIST on day 67, died from TMA/VOD
9	45/F	Normal	CR1	0	Identical related	No		II	Extensive	alIve in CR1	966	
10	50/F	t(9:22)(q34;q11)	CR1	0	Identical related	Day+92		0	No	Dead	421	Leukemia
11	23/F	Others	First relapse	1	Matched unrelated	No		0	Limited	Alive in CR2	828	
12	35/M	t(9:22)(q34;q11)	First relapse	4	Matched unrelated	No		II	No	Death in CR1	130	Pulmonary hemorrhage after *Pseudomonas* pneumonia
13	56/M	t(9:22)(q34;q11)	Primary refractory	1	Identical related	Day+712		III	Extensive	Alive with disease	734	
14	64/F	Normal	CR1	0	Identical related	No		0	Extensive	Alive in CR1	516	
15	46/M	t(9:22)(q34;q11)	CR1	0	Matched unrelated	No		I	Extensive	Died in CR1	162	MRSA pneumonia, sepsis
16	53/F	Hypodiploid	First relapse	0	Identical related	No		III	Unavailable	Death in CR2	167	Endotoxic shock
17	55/M	Normal	CR2	0	Identical related	Day+84	Relapse	0	Extensive	died in CR3	136	Severe GVHD following DLI
18	67/M	t(9:22)(q34;q11)	CR1	1	Identical related	No		I	Extensive	Died in CR1	176	Respiratory failure due to idiopathic pulmonary syndrome
19	55/F	Normal	Primary refractory	1	Identical related	No		0	Extensive	Alive in CR1	333	
20	59/M	t(9:22)(q34;q11)	CR1	1	Identical related	No		0	Extensive	Alive in CR1	347	
21	52/F	Others	CR1	NE	Matched unrelated	No		I	NE	Died in CR1	68	Respiratory failure due to diffuse alveolar damage
22	60/F	t(1;19)(q23;p13.3)	First relapse	2	Mismatched related			IV	NE	Death	54	Severe GVHD after FK tapering for refractory disease
23	60/F	t(9:22)(q34;q11)	CR2	0	Identical related	Day+99	Relapse	II	Extensive	Dead	148	Leukemia
24	33/F	Others	Second relapse	1	Mismatched unrelated	No		IV	Limited	Death in CR3	186	Fungal pneumonia

Table 2 Continued

Patient	*Age/ sex*	*Karyotype*	*Status before RIST*	*Performance status*	*Donor*	*Relapse*	*Reason for DLI*	*Acute GVHD*	*Chronic GVHD*	*Present status*	*Follow-up, days*	*Cause of death*
25	27/M	Normal	CR2	1	Matched unrelated	No		II	Limited	Alive in CR2	257	
26	59/F	t(9:22)(q34;q11) +complex	Primary refractory	0	Matched unrelated	No		IV	No	Death in CR1	125	Liver GVHD followed by multiple organ failure
27	65/F	t(9:22)(q34;q11)	Second relapse	2	Mismatched related	Day+138		0	No	Death	182	Leukemia
28	55/F	t(9:22)(q34;q11)	CR1	1	Identical related	No		0	No	Alive in CR1	166	
29	17/M	Others	Primary refractory	3	Identical related			0	NE	Death	40	Leukemia in CNS
30	29/F	Normal	Third relapse	1	Identical related	Day+113		II	No	Alive with disease	148	
31	37/F	Complex	CR2	0	Matched unrelated	Day+86		I	No	Alive with disease	131	
32	56/F	Normal	CR1	1	Identical related	Day+99		0	No	Dead	178	Leukemia
33	59/F	Normal	CR1	0	Mismatched related	No		III	No	Alive in CR1	104	

M = male; F = female; CR = complete remission; NE = not evaluated; DLI = donor lymphocyte infusion; GVHD = graft-versus-host disease; CNS = central nervous system, TMA = thrombotic microangiopathy; VOD = veno-occlusive disease.

Association between GVHD and OS

Among the 21 patients who survived without disease progression longer than 100 days, presence of grades II–IV acute GVHD tended to show better PFS compared with those without it; however, the difference was marginal (relative risk 0.45,. 95% CI 0.18–1.16, $P = 0.10$) (Figure 4).

DLI

DLI was undertaken in six patients. Patient 4 received prophylactic DLI and achieved durable molecular remission until she died of bronchitis obliterans at 13.5 months after RIST. The remaining five patients underwent DLI following recurrence of ALL. Two patients (Patients 3 and 8) received DLI followed by second RIST. Patient 8 underwent second RIST 38 days after DLI, and died from progressive disease 29 days after second RIST. Patient 3 received DLI for central nervous system (CNS) relapse, and underwent second RIST. She relapsed again in the CNS and bone marrow, and died of disease progression 8.7 months after second RIST.

Response to DLI was evaluated in the remaining three patients (Patients 2, 17, and 23). One patient responded to DLI, and two died of acute GVHD. Patient 2 received DLI for emerging extramedullary disease. The lesion was controlled using local irradiation and DLI. However, ALL recurred in the BM and the patient experienced grade IV acute GVHD that eventually proved fatal. In Patient 17, peripheral blasts disappeared after DLI, but the patient died of gastrointestinal GVHD. Patient 23 died of disease progression without any response to DLI.

Prognostic factors for PFS

In univariate analyses, no variables were identified as significant prognostic factors for PFS (Table 3). Multivariate analysis was discontinued due to the lack of associated factors from univariate analyses.

Discussion

GVL effects against ALL after allo-SCT have been discussed for almost 15 years.[31,32] A small but significant proportion of patients with advanced ALL achieve durable remission following RIST. Of the 14 patients transplanted in relapse, 12 achieved durable remission, and molecular remission was confirmed in four of the seven patients with t(9;22)(q34;q11) in this study. Considering that combination chemotherapy is usually ineffective at producing prolonged survival in patients with advanced ALL, the long-term PFS after allo-SCT suggests that durable allogeneic immune reactions continue to suppress leukemic progression.

Optimal reduced-intensity regimens remain unclear for RIST for ALL. Even after achievement of CR following allo-SCT, patients with ALL display high levels of minimal residual disease associated with increased relapse rates.[33] Most physicians believe that GVL effects are insufficient or may not have enough time to arrest advanced ALL, and

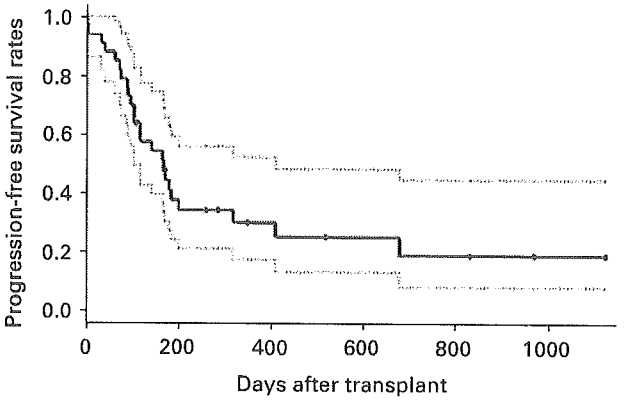

Figure 1 Progression-free survival. Probability of 2-year PFS and OS was 18.6% (95% confidence interval (CI), 2.4–34.9%) and 29.7% (95% CI, 11.7–47.7%), respectively. Broken lines show 95% CI. PFS at 3 years was 18.6% (95% confidence interval, 2.4–34.9%).

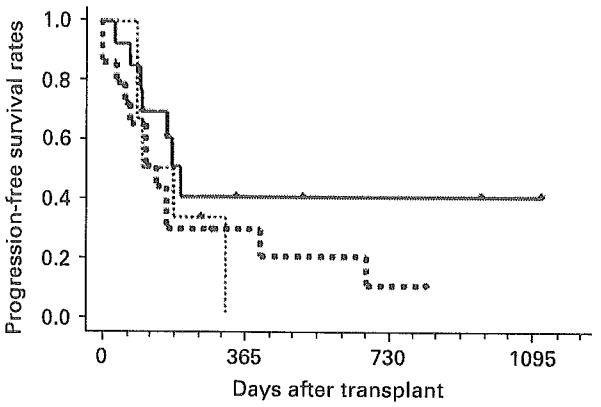

Figure 3 Association between progression-free survival (PFS) rates and disease status at RIST. Actuarial 1-year PFS rates were 30.6% (95% CI, 7.7–53.5%) for the 19 patients transplanted in CR1/CR2 and 28.6% (95% CI, 4.9–52.2%) for the 14 patients transplanted in relapse or induction failure ($P = 0.26$). Solid line indicates patients in CR1 and large broken line indicates patients in CR2. Small broken line indicates the others.

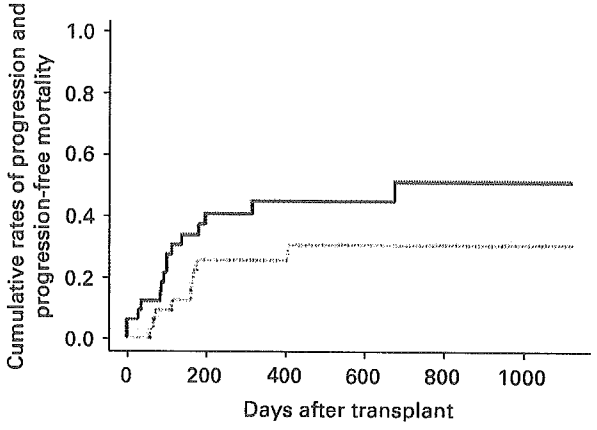

Figure 2 Cumulative rates of progression and progression-free mortality. Cumulative incidence of progression at 3 years was 50.9%. Cumulative incidence of nonprogression mortality at 3 years was 30.4%. Solid line indicates progression and broken line indicates progression-free mortality.

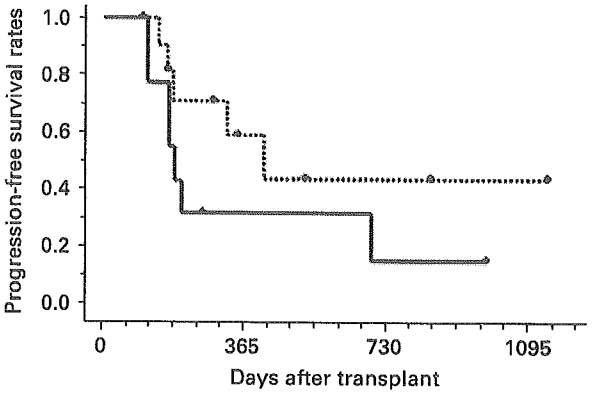

Figure 4 Influence of acute GVHD on progression-free survival (PFS) rates. Among the 21 patients who survived without disease progression longer than 100 days, presence of grades II–IV acute GVHD tended to show better PFS compared with those without it; however, the difference was marginal (relative risk 0.45, 95% CI 0.18–1.16, $P = 0.10$). Solid line indicates patients with grades II–IV acute GVHD ($n = 10$). Broken line indicates patients with acute GVHD less than grade II ($n = 11$).

that preparative regimens should be more intense in RIST for ALL compared with chronic-phase chronic myeloid leukemia or low-grade malignant lymphoma. Purine analog-based regimens were mostly used in this study, and chemotherapeutic agents such as melphalan, busulfan and 4–8 Gy TBI were added in 28 patients. These regimens might have been beneficial for establishing durable engraftment in RIST for advanced ALL, and might have contributed to temporary control of disease. The role of additional chemoradiotherapy needs to be further investigated in RIST for ALL.

Optimal timing of RIST for ALL remains unknown. Estimated 2-year PFS rates after RIST for high- and low-risk ALL were 8 and 42%, respectively. Outcomes for RIST are dismal unless therapy is given in CR1 (Figure 3). These findings are comparable to previous reports on conventional allo-SCT for ALL.[34,35] Disease status remains an important prognostic factor in RIST and decision-making as to when to proceed with allo-SCT in patients with ALL is difficult.

Response rates to DLI in ALL patients were low,[12,13] suggesting a limited GVL effect for ALL, especially after hematologic relapse. Only early intervention before clinical relapse can improve prognosis for these patients, by inducing GVL effects.[13,36] In our study, DLI induced remission in one of five patients who relapsed following RIST, accompanied by severe GVHD. Another two patients died of leukemic progression, and the other two patients died of GVHD following DLI. However, some patients dramatically benefit from DLI such as Patients 2 and 17 in our study, and the one reported by Slavin *et al.*[7] Many publications suggest that the efficacy of DLI may be improved by activation of donor lymphocytes using *in vitro* or *in vivo* interleukin-2 with no prohibitive GVHD.[37] Activation of donor lymphocytes is a possible future approach in an attempt to amplify the already well-documented GVL effect in ALL.

Table 3 Risk factors for overall survival following allogeneic hematopoietic stem-cell transplantation

Factors	*Hazard ratio*	*95% Confidence interval*	*P*
Univariate analysis			
Pre-transplant factors			
Age $\geqslant 50$ *vs* <50 years	1.1	0.47–2.57	0.83
Phl *vs* others	1.48	0.66–3.31	0.34
Previous SCT (+) *vs* (−)	0.51	0.15–1.73	0.28
CR1 *vs* others	0.53	0.22–1.28	0.16
CR12 *vs* others	0.64	0.28–1.42	0.27
PS34 *vs* PS012	1.7	0.50–5.78	0.39
PB *vs* BM	0.82	0.34–2.00	0.66
Matched related *vs* others	0.78	0.34–1.76	0.54
GVHD prophylaxis single *vs* combined	0.58	0.22–1.57	0.29
Post transplant factor			
GVHD II–IV present *vs* absent	2.2	0.86–5.62	0.10

Phl = Philadelphia chromosome 1; SCT = stem cell transplantation; CR1 = first complete remission; CR12 = first or second complete remission; PS = ECOG performance status; PB = peripheral blood stem cells, BM = bone marrow stem cells; GVHD = graft-versus-host disease.

Two basic types of complications are associated with allo-SCT: regimen-related toxicity and GVHD. Nonrelapse mortality rate within 100 days of transplantation was 6.4% in this study. Reduced-intensity regimens cause less organ damage, contributing to lower rates of TRM. These findings are comparable to previous reports.[14,15,38] GVHD represents another significant concern after RIST,[39] which is frequently complicated by infections.[40] Rates of acute and chronic GVHD were 45 and 64%, respectively. The balance between GVHD and GVL is delicate in allo-SCT. Augmentation of GVHD prophylaxis may hamper GVL effects, and leukemic cells cannot be eradicated by reduced-intensity conditioning alone. This study failed to show meaningful advantages of GVHD in preventing relapse of ALL following RIST, while GVHD remains the leading cause of TRM. At present, allo-SCT recipients receive uniform GVHD prophylaxis irrespective of the risk of underlying disease or patient condition. Intensification of GVHD prophylaxis is at least beneficial in RIST for low-risk ALL. Management of GVHD prophylaxis should be optimized considering risk of underlying disease and patient condition.

Our study indicates that RIST is worth considering for further intense evaluation. The technique may offer the best chance for hematologic remission and prolonged survival, given the poor prognosis after conventional chemoradiotherapy. However, this retrospective study was too small to provide definitive conclusions regarding RIST for ALL. Since our study included heterogeneous patient group, centers, and conditioning approaches, interpretation of the results requires caution. Further investigation and data are awaited to determine the indications and optimal transplantation procedures for ALL.

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bjh research paper

Evaluation of cytomegalovirus-specific T-cell reconstitution in patients after various allogeneic haematopoietic stem cell transplantation using interferon-γ-enzyme-linked immunospot and human leucocyte antigen tetramer assays with an immunodominant T-cell epitope

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Summary

Cytomegalovirus (CMV) infection is a major complication for patients who received allogeneic haematopoietic stem cell transplantation (HSCT). Accurate monitoring of CMV-specific T-cell reconstitution is required for appropriate decision on treatment, such as anti-viral drugs, which have adverse effects. Although human leucocyte antigen (HLA) tetramer and interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assays have been used to measure CMV-specific T cells, detailed comparison of these assays and kinetics of anti-CMV T-cell reconstitution between reduced-intensity transplantation (RIST) and conventional HSCT has not yet been performed. In this study, we performed prospective comparative monitoring of CMV-specific T cells using HLA tetramer and IFN-γ-ELISPOT assays with a single immunodominant CMV_{495} peptide in 28 HLA-A*0201 and 9 HLA-A*0206 patients after various allogeneic HSCTs. The IFN-γ-ELISPOT assay was more sensitive for evaluation of functional T cells than the HLA tetramer assay, and CMV-specific T cells were reconstituted earlier in patients who received RIST without anti-thymocyte globulin (ATG) than those receiving RIST with ATG or conventional HSCT. The threshold level for protection from CMV reactivation was estimated as over 1×10^6 cells/l peripheral blood with the IFN-γ-ELISPOT assay. These results demonstrate that the IFN-γ-ELISPOT assay with CMV_{495} provides more accurate evaluation on CMV immunity in HLA-A*0201 and -A*0206 patients, and may be useful for determining timing of various treatments.

Keywords: cytomegalovirus, haematopoietic stem cell transplantation, interferon-γ-enzyme-linked immunospot, human leucocyte antigen tetramer, reduced-intensity transplantation.

Reactivation of latent cytomegalovirus (CMV) leads to an increased risk of life-threatening complications in immunocompromised hosts, including those receiving haemopoietic stem cell transplantation (HSCT) (Reusser *et al*, 1991; Li *et al*, 1994; Boeckh *et al*, 2003). CMV-specific T cells play an important role in the control of CMV reactivation (Reusser *et al*, 1991; Li *et al*, 1994; Walter *et al*, 1995; Einsele *et al*, 2002; Boeckh *et al*, 2003; Peggs *et al*, 2003). Although CMV antigenaemia (Nichols *et al*, 2001; Kanda *et al*, 2002) or polymerase chain reaction (PCR)-guided (Einsele *et al*, 1995) preemptive ganciclovir therapy have been shown to be effective for the prevention of CMV disease, ganciclovir administration results in an increased risk of cytopenia or late onset of CMV disease. Thus, accurate monitoring of CMV-specific T-cell recovery is important for ganciclovir administration timing (Cwynarski *et al*, 2001; Gratama *et al*, 2001; Ozdemir *et al*, 2002; Maris *et al*, 2003; Mohty *et al*, 2004).

The *in vitro* induction of CMV-specific T cells by stimulating peripheral blood mononuclear cells (PBMC) with viral infected cells has previously been performed (Reusser *et al*,

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1991; Li *et al*, 1994; Walter *et al*, 1995). Although this method provides whole anti-viral T-cell responses restricted by the patients human leucocyte antigens (HLAs), it is labour intensive and lacks quantitative analysis. The structural, major, late CMV matrix proteins, pp65 (Solache *et al*, 1999; Kuzushima *et al*, 2001) and IE (Hebart *et al*, 2003), were identified as immunodominant CMV antigens for cytotoxic T lymphocytes (CTL), and their T-cell epitopes have been identified. Quantitative evaluation of CMV-specific T cells using the HLA tetramer (Cwynarski *et al*, 2001; Gratama *et al*, 2001; Ozdemir *et al*, 2002; Mohty *et al*, 2004) and interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assays (Mohty *et al*, 2004) has been recently introduced. However, evaluation of the immunodominant nature of various CMV T-cell epitopes and direct comparison of these two assays with a single immunodominant peptide has not yet been performed in patients who have undergone HSCT.

Reduced-intensity transplantation (RIST) is increasingly applied for patients who are not eligible for conventional myeloablative HSCT (Mohty *et al*, 2000; Kanda *et al*, 2001; Chakrabarti *et al*, 2002; Junghanss *et al*, 2002; Nakai *et al*, 2002). The RIST regimens rely on immunosuppressive agents for prevention of graft rejection and acute graft-*versus*-host disease (GVHD), and sometimes use potent immunosuppressive drugs, such as antithymocyte globulin (ATG) (Mohty *et al*, 2000, 2004; Kanda *et al*, 2001, Nakai *et al*, 2002) or Campath-1H (Chakrabarti *et al*, 2002), which delay immune reconstitution after HSCT. Thus, it is important to investigate reconstitution of CMV-specific T cells among these different HSCT protocols.

In this study, we first determined the immunodominancy of the previously identified HLA-A*0201-restricted CMV peptides (Solache *et al*, 1999) among patients with various HLA-A2 subtypes. CMV-specific T cells were prospectively monitored with the immunodominant $pp65_{495-503}$, using both HLA tetramer and IFN-γ-ELISPOT assays in patients who received RIST with or without ATG or conventional HSCT. The IFN-γ-ELISPOT assay was found to be more sensitive for monitoring functional CMV-specific T cells in aiding the timing of ganciclovir administration and withdrawal, and that RIST without ATG showed an advantage for the earlier recovery of CMV immunity than other HSCT protocols.

Materials and methods

Patients and HSCT protocols

This research was approved by the Committee on Ethical and Clinical Investigation of the National Cancer Centre Hospital (NCCH). Informed consent was obtained from recipients and donors before study entry according to the Declaration of Helsinki. Thirty-seven patients (28 HLA-A*0201 and 9 HLA-A*0206) who underwent allogeneic peripheral blood stem cell transplantation (PBSCT) from HLA-matched siblings at NCCH between April 2001 and August 2003, were evaluated in this study (Table I). The HLA type of the donors and recipients was determined by PCR using sequence-specific primers. The preparative regimens were classified into three groups; (i) conventional regimen using 12 Gy of total body irradiation and 120 mg/kg of cyclophosphamide ($n = 5$) or the combination of 16 mg/kg oral busulphan and 120 mg/kg of cyclophosphamide ($n = 4$), (ii) RIST (8 mg/kg busulphan and 180 mg/m^2 fludarabine) with ($n = 11$) or (iii) without ($n = 17$) 5 mg/kg of rabbit ATG. Heparin-treated blood samples were taken at 1-week intervals after engraftment when

Table I. Characteristics of patients and transplant protocols.

Patient characteristics	No. of patients
Total, *n*	37
Median age, years (range)	44·8 (9–68)
Sex (male/female)	26/11
Disease	
Acute myeloid leukaemia	9
Acute lymphoblastic leukaemia	2
Myelodysplastic syndrome	5
Chronic myeloid leukaemia	5
Non-Hodgikin lymphoma	6
Multiple myeloma	1
Severe anaplastic anaemia	2
Solid tumour	7
Conditioning regimen	
Conventional	9
RIST ATG (+)	11
RIST ATG (−)	17
Source of stem cells	
Peripheral blood stem cells	37
CMV serologic status donor/recipient	
Positive/positive	32
Positive/negative	3
Negative/negative	2
HLA status	
HLA-A*0201	28
HLA-A*0206	9
Acute GVHD	
0-I	18
II	6
III-IV	13
Steroid use (mg/kg)	
0	20
1	11
2–10	6
Donor type	
Matched family	30
Mismatched family	7
Methods of GVHD prophylaxis	
CSP alone	18
CSP and MTX	19
Follow up day, median (range)	145·4 (42–325)

GVHD, graft-*versus*-host disease; CSP, cyclophosphamide; MTX, methotrexate; RIST, reduced-intensity transplantation; HLA, human leucocyte antigen.

patients were hospitalised. HLA tetramer, ELISPOT, and antigenaemia assays were performed using the blood samples taken on the same day.

Evaluation of CMV infection

Pre-transplant sera from patients and donors were tested for CMV-specific immunoglobulin G (IgG) antibody (Ab) using an enzyme-linked immunosorbent assay (ELISA). CMV antigenaemia was evaluated using the previously described method (Gondo *et al*, 1994). Briefly, $1{\cdot}5 \times 10^5$ peripheral blood leucocytes were fixed on slides and incubated with anti-CMV pp65 monoclonal antibody (mAb) C7 conjugated with horse radish peroxidase (Teijin, Tokyo, Japan), stained using the direct immunoperoxidase method, and counted under a light microscope. The results are presented as numbers of positive cells per 50 000 leucocytes.

Induction of CMV-specific T cells with the synthesised CMV peptides

The CMV $pp65_{495-503}$ (NLVPMVATV), $pp65_{14-22}$ (VLGPISGHV), $pp65_{120-128}$ (MLNIPSINV) and melanoma antigen $gp100_{280-288}$ (YLEPGPVTA) were synthesised with a multiple peptide synthesiser (ACT 396; Advanced ChemTech, Louisville, KY, USA) using a standard solid-phase method with Fmoc chemistry, purified using reverse-phase high performance liquid chromatography (HPLC), and confirmed by mass spectral analysis. The purity of the peptides was over 90%. Two million PBMC were stimulated with 4 μg/ml of the CMV peptides in a well of 24-well plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% human pooled AB serum (HS), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C, and 100 IU/ml of recombinant human interleukin-2 (IL-2) (Shionogi, Osaka, Japan) was added on day 2. On days 7 and 14, the recovered cells were re-stimulated with irradiated peptide-pulsed autologous PBMC at a responder to stimulator ratio of 4:1, and IL-2 was added on day 9 and 16.

Production of HLA tetramers and analysis of specific T cells

Human leucocyte antigen-A*0201 tetramers were produced using previously described methods (Altman *et al*, 1996). Briefly, recombinant HLA-A*0201 heavy chains with the C terminal modification containing a substrate sequence for biotinylating enzyme BirA, and β_2-microglobulins were produced in *Escherichia coli* transformed with the relevant plasmids. Monomeric HLA-peptide complexes were generated by mixing the HLA heavy chains, β_2-microglobulins and antigenic peptides, then biotinylated by using recombinant BirA enzymes (Avidity, Denver, CO, USA) in the presence of biotin, and purified using gel filtration and anion exchange chromatography. HLA tetramers were then produced by mixing the biotynylated HLA-A2/peptide complexes with streptavidin-phycoerythrin (PE) (BD PharMingen, San Diego, CA, USA) at a molar ration of 10:1.

The CMV-specific CD8+ T cells in peripheral blood were evaluated with the HLA tetramers as follows: PBMC (10^6) were incubated with 2 μg/mL of PE-labelled HLA tetramers in 96-well plates for 30 min at 37°C, washed once with 200 μL of phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS), stained with fluorescein isothiocyanate (FITC)-labelled anti-CD8 mAb (T8-FITC; Beckman Coulter, Miami, FL, USA) for 1 h at 4°C, washed three times, and stained with 1 μg/ml 7-aminoactinomycin D (7-AAD) (Beckman Coulter) for 20 min at room temperature in 200 μL 5% FCS PBS. Stained cells were immediately analysed on a FACSCalibur using CELLQUEST software (Becton-Dickinson, Mountain View, CA, USA).

The frequency of CMV-specific T cells measured with the HLA tetramer in 17 CMV seropositive healthy individuals expressing either HLA-A*0201 or HLA-A*0206 was 0·76 ± 0·78% (mean ± 2SD) (range; 0·09–2·88%) of peripheral blood CD8+ T cells. Background staining obtained with the melanoma antigen $gp100_{280-288}$/HLA-A2 tetramer was 0·02%. The lower limit of detection by the CMV$pp65_{495}$ HLA tetramer was 0·02%, the lowest detection level of HLA tetramer positive cells by flow cytometry. The absolute number of the HLA tetramer positive T cells was calculated as: [lymphocyte count/peripheral blood (L)] × (numbers of HLA tetramer positive cells/total numbers of lymphocytes analysed by flow cytometry).

Measurement of IFN-γ producing CMV-specific T cells using ELISA or ELISPOT assay

The B-cell/T-cell hybrid cell line 174 CEM.T2 (T2) and COS-7 transfected with a plasmid expressing HLA-A*0206, were maintained in RPMI 1640 containing 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Fifty thousands T cells were cultured with either 1×10^5 HLA-A*0201 T2 cells or HLA-A*0206 transfected COS-7 cells pulsed with 5 μg/ml peptides for 16 h at 37°C in 2% human serum RPMI 1640 medium. IFN-γ in the culture supernatants was measured using IFN-γ-ELISA as previously described (Kawakami *et al*, 1994). The IFN-γ-ELISPOT assay was performed as previously described (Kuwana *et al*, 2003). Briefly, PBMC ($2{\cdot}5$ or 1×10^5 per well) were stimulated with 5 μg/ml of the peptides, and incubated at 37°C for 16 h in 10% human serum IMDM medium, transferred to the multiscreen nitrocellulose 96-well immunoplate (Millipore, Bedford, MA, USA) coated with 15 μg/ml of anti IFN-γ-mAb (1-D1K; Mabtech, Stockholm, Sweden), and incubated at 37°C for 16 h. After 10 washes, it was mixed with 1 μg/ml of anti-IFN-γ biotinylated mAb (7-B6-1; Mabtech, Stockholm, Sweden) for 2 h at room temperature, washed 10 times with PBS, incubated with streptavidin-bound alkaline phosphatase (diluted 1 in 1000 in filtered) for 1 h at room temperature. After six washes with

PBS, IFN-γ spots were visualised by incubation with nitro blue tetrazolium/5-bromo-4-chloro-indolyl phosphate. Numbers of spot-forming cells (SFC) were counted using a computer-based evaluation system (KS-ELISPOT; Carl Zeiss, Hallbergmoos, Germany).

The frequency of CMV-specific T cells with the IFN-γ-ELISPOT assay was 84·7 ± 106·8 (range; 1–298·3) SFC/$2·5 \times 10^5$ PBMC (mean ± 2SD) in 17 CMV seropositive healthy individuals expressing either HLA-A*0201 or HLA-A*0206. Background counts obtained with the $gp100_{280}$ was 5·3 SFC/$2·5 \times 10^5$ PBMC, the lower limit of detection in this study. The absolute number of CMV-specific T-cell activity was calculated by the following formula; [lymphocyte and monocyte counts/peripheral blood (L)] × (numbers of IFN-γ positive spots/total number of added PBMC).

Statistical analysis

Spearman rank test was used to evaluate the correlation among the frequencies of IFN-γ producing cells, HLA tetramer-positive cells, and CMV antigenaemia. Paired and unpaired *t*-test were used for comparisons of the IFN-γ-ELISPOT and HLA tetramer assays as well as three different HSCT conditioning regimens. Kaplan–Meier analysis was applied for analysis of the relationship between the conditioning regimens and the day when CMV-specific T cells reached the threshold level after transplantation. Log-rank test was used to measure significant difference of cumulative rates of patients who reached the threshold level among patients with various HSCTs. All tests were two-tailed and were considered significant when $P < 0·05$.

Results

*The CMV $pp65_{495–503}$ peptide is an immunodominant in both HLA-A*0201 and HLA-A*0206 individuals*

To evaluate the immunodominant nature of the previously identified CMV pp65 peptides restricted by HLA-A*0201 (Solache *et al*, 1999), including $pp65_{495–503}$ (NLVPMVATV), $pp65_{14–22}$ (VLGPISGHV), and $pp65_{120–128}$ (MLNIPSINV) in the Japanese population, whose popular HLA-A2 subtypes are HLA-A*0201 (10·6%), HLA-A*0206 (8·4%) and HLA-A*0207 (4·0%) (Tokunaga *et al*, 1997), we performed *in vitro* induction of CMV-specific T cells by stimulation with these three peptides from PBMC of 8 HLA-A*0201, 6 HLA-A*0206 and 3 HLA-A*0207, CMV-seropositive healthy donors. After 2–3 *in vitro* stimulations, CMV peptide-specific T cells were detected with IFN-γ release assay and HLA-A*0201 tetramer assay. CMV_{495}-specific T cells were induced from seven of eight HLA-A*0201 positive and all six HLA-A*0206 positive donors, while they were not induced or only detected in one case with other peptides or HLA-A*0207 donors (data not shown). It was noted that the CMV_{495}/HLA-A*0201 tetramer was able to specifically visualise not only HLA-A*0201 restricted, but also HLA-A*0206 restricted, CMV_{495}-specific T cells, indicating its use for immuno-monitoring HLA-A*0206 patients (data not shown). These results demonstrated that CMV_{495} is an immunodominant CMV epitope for both HLA-A*0201 and -A*0206 individuals. Therefore, we decided to use CMV_{495} to monitor CMV-specific T cells in patients after allogeneic HSCT, as it is important to use immunodominant epitopes to estimate total CMV immunity from the results with a single T-cell epitope.

The IFN-γ-ELISPOT assay is more sensitive to detect functional CMV-specific T cells than the HLA tetramer assay

Using IFN-γ-ELISPOT and HLA tetramer assays with the CMV_{495} peptide, we performed immuno-monitoring of CMV immunity in the patients who received various HSCTs. A significant correlation was observed between the frequencies obtained with the HLA tetramer and IFN-γ-ELISPOT assays ($P < 0·001$). Absolute numbers of CMV-specific T cells determined with the HLA tetramer was higher than those obtained with the IFN-γ-ELISPOT assay probably because of detection of functional T cells with the ELISPOT assay among all CMV_{495}-specific T cells detected with the HLA tetramer.

The day that CMV-specific T cells were first detected after various HSCTs were evaluated in the HLA-A*0201 or -A*0206 positive, CMV seropositive patients. The patients who received steroid therapy before the first detectable time point of CMV-specific T cells after HSCTs were excluded from this analysis. The mean ± 2SD of first detected day was 55 ± 31 (range; 14–113 d) with the tetramer and day 37 ± 31 (13–113 d) with the ELISPOT, respectively (Fig 1, $P < 0·001$), indicating that the IFN-γ-ELISPOT assay was more sensitive for detection of CMV-specific T cells than the HLA tetramer assay.

Early reconstitution of CMV-specific T cells in patients who received RIST without ATG

Recovery of CMV-specific T cells was then compared among the HLA-A*0201 or -A*0206 positive, CMV seropositive patients who received various allogeneic HSCTs using the HLA tetramer and IFN-γ-ELISPOT assays. With the tetramer or ELISPOT, CMV-specific CD8+ T cells were first detected on day 36 ± 16 (mean ± 2SD) (14–65 d), or day 22 ± 6 (14–29 d) after HSCT in the patients who received RIST without ATG, on day 62 ± 32 (23–104 d), or day 35 ± 4 (23–56 d) in the patients who received RIST with ATG, or on day 79 ± 39 (14–113 d), or day 73 ± 19 (13–113 d) in the patients who received conventional HSCT, respectively (Fig 2A, B). With the ELISPOT, the reconstitution of CMV-specific T cells was profoundly delayed in the patients who received conventional HSCT than those who received RIST with ($P = 0·03$) or without ATG regimen ($P = 0·001$). In the patients with RIST, ATG administration significantly delayed recovery of CMV-specific T cells ($P = 0·007$).

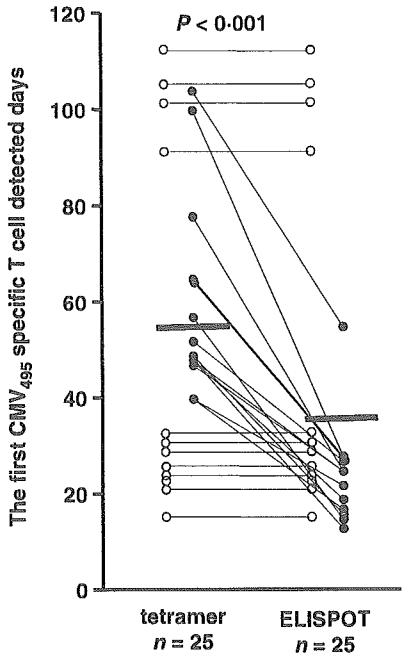


Fig 1. Earlier detection of cytomegalovirus (CMV)-specific T cells with the interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assay than human leucocyte antigen (HLA) tetramer assay in patients after haematopoietic stem cell transplantations (HSCT). Data points indicate the first detected day of CMV_{495}-specific T cells in each patient (○ and ●) and the mean value (—). CMV-specific T cells was detected significantly earlier with the IFN-γ-ELISPOT assay than the HLA tetramer assay in patients after HSCTs ($P < 0{\cdot}001$, paired t-test).

Threshold level of CMV-specific T cells for protection from CMV reactivation

To determine the level of CMV-specific T cells sufficient to protect CMV reactivation and infection, correlation between CMV-specific T-cell frequency and CMV antigenaemia was examined (Fig 3A). CMV antigenaemia was not detected in the patients who had absolute CMV-specific T cells over 1×10^7/L in the tetramer or over 1×10^6/L in the ELISPOT assay (Fig 3A), indicating that these threshold levels may be useful in aiding decisions regarding treatment for CMV reactivation and infection. The cumulative rate of patients who reached the threshold level (1×10^6/L) of CMV-specific T cells measured with the ELISPOT after various HSCTs was shown in Fig 3B. The patients who received RIST without ATG reached the threshold level significantly earlier than those who received RIST with ATG, or conventional HSCT.

Discussion

Development of reliable methods for the evaluation of immune status against CMV is clinically important for the management of CMV reactivation in transplant recipients. In this study, using the identified CMV_{495} epitope, which is strongly immunogenic for HLA-A*0201 or HLA-A*0206 patients, we compared various allogeneic HSCTs for the reconstitution of CMV immunity using both IFN-γ-ELISPOT and HLA tetramer assays. The frequency of CMV-specific T cells detected with the tetramer was, on average, 1·5-fold higher than that with the ELISPOT, probably because of the

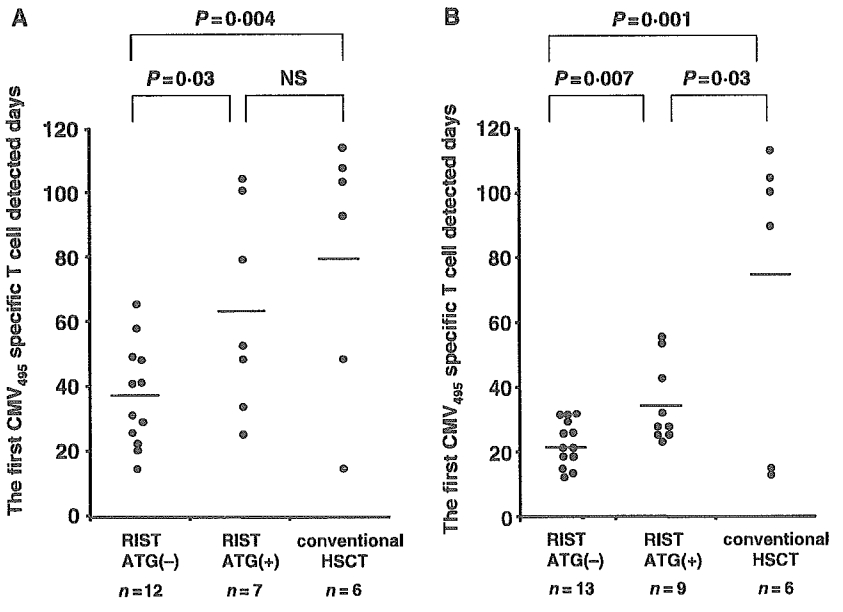
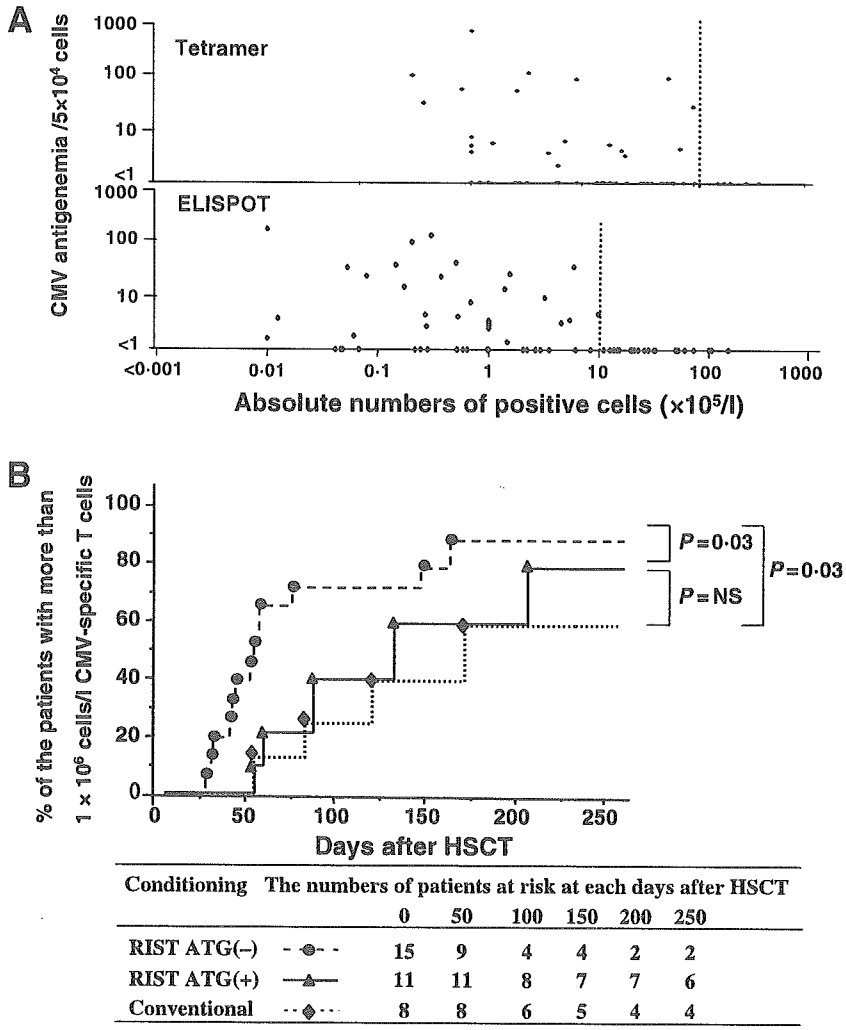


Fig 2. Earlier reconstitution of cytomegalovirus (CMV)-specific T cells in patients who received reduced-intensity transplantation (RIST) without anti-thymocyte globulin (ATG) than those who received RIST with ATG or conventional haematopoietic stem cell transplantation (HSCT). The first detected days of CMV_{495}-specific T cells determined with HLA tetramer assay (A) and interferon-γ-enzyme-linked immunospot assay (B), are shown among patients who received RIST without/with ATG and conventional HSCT. Data points indicate each patient (●) and the mean value (—).



Conditioning	The numbers of patients at risk at each days after HSCT					
	0	50	100	150	200	250
RIST ATG(–)	15	9	4	4	2	2
RIST ATG(+)	11	11	8	7	7	6
Conventional	8	8	6	5	4	4

Fig 3. (A) The threshold level (dotted line) of cytomegalovirus (CMV)-specific T cells for efficient protection from CMV antigenaemia. Data points show each patient's status of CMV antigenaemia and absolute numbers of CMV_{495}-specific T cells evaluated with the human leucocyte antigen (HLA) tetramer assay and interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assay was more sensitive for detection of CMV-specific T cells than the HLA tetramer assay (ELISPOT assay). CMV reactivation was not observed when absolute numbers of CMV_{495}-specific T cells were over 1×10^7/l in the HLA tetramer assay, or over 1×10^6/l IFN-γ-ELISPOT positive cells. (B) Earlier reconstitution of CMV immunity to the threshold protection level in the patients who received reduced-intensity transplantation (RIST) without ATG compared with those who received RIST with ATG or conventional HSCT. Kaplan–Meier analysis was employed to evaluate the difference of days for absolute CMV_{495}-specific T cells to reach the threshold protection level, over 1×10^6/l IFN-γ-ELISPOT positive cells among patients who received RIST without/with ATG and conventional HSCT.

detection of only functional T cells with IFN-γ-ELISPOT assay. Similar observations were reported in CMV and HIV infections (Goepfert *et al*, 2000; Sun *et al*, 2003; Mohty *et al*, 2004). This discrepancy between the results obtained by two assays appears to be exaggerated, particularly when CMV-specific T cells are rapidly expanding in response to CMV antigenaemia. It was reported that ratio of tumour necrosis factor-α-producing T cells, measured by intracellular staining per HLA tetramer positive T cell, was lower in the HSCT patients who experienced CMV antigenaemia than those without CMV antigenaemia. HLA tetramer-positive T cells which do not produce IFN-γ have been reported to have a greater proliferative capacity (Sallusto *et al*, 1999). Detectable cells in this phase may be less differentiated, CCR7-positive, $CD45RA^{low}$ central memory T cells which may later differentiate to CCR7 negative effector memory ($CD45RA^{low}$) and terminally differentiated effector ($CD45RA^{high}$) cells capable of producing IFN-γ.

Sensitivity for the detection of CMV-specific T cells was higher with the ELISPOT (detection limit is $1/1 \times 10^5$ PBMC) than with the tetramer, mainly because of the detection threshold (approximately 0·02% of CD8+ T cells) defined by flow cytometric analysis. We compared the first detected day of CMV-specific T cells after various HSCTs using both assays, and found that the first detectable day was significantly earlier with ELISPOT than the tetramer assay, indicating that IFN-γ-ELISPOT assay is recommended for sensitive measurement of functional CMV-specific T cells, if it can be performed in the institution.

We then compared reconstitution of CMV-specific T cells among various HSCT protocols, including RIST with or without ATG, and conventional HSCT. Although the recovery of CMV-specific T cells and the incidence of CMV infection after RIST have previously been reported from several groups, including ours (Mohty *et al*, 2000; Kanda *et al*, 2001; Chakrabarti *et al*, 2002; Junghanss *et al*, 2002; Nakai *et al*, 2002; Maris *et al*, 2003), detailed comparison for CMV-specific T-cell reconstitution among different HSCT protocols using a single immunodominant peptide, has not been performed. The reconstitution of CMV immunity was significantly earlier in the patients who received RIST without ATG than RIST with ATG or conventional HSCT. Threshold levels of the CMV immunity for effective protection of CMV reactivation were 1×10^6/l with the ELISPOT assay and 1×10^7/l with the tetramer assay as a total CMV immunity, which are consistent with previous reports (Cwynarski *et al*, 2001; Gratama *et al*, 2001).

Anti-thymocyte globulin inhibited the early recovery of functional IFN-γ-producing and HLA tetramer-positive CMV-specific T cells. Eight of eleven patients who received RIST with ATG developed early CMV antigenaemia on day 27, while 6 of 17 patients who received RIST without ATG developed early CMV antigenaemia on day 37. Similar observations were reported in the RIST patients who received ATG using IFN-γ-ELISPOT assay with CMV lysates, although they could not conclusively show a negative effect of ATG on CMV-specific T cells because of small number of samples (Mohty *et al*, 2000). A high CMV infection rate after Campath-1H-based conditioning was also reported (Chakrabarti *et al*, 2002). The use of strongly immunosuppressive ATG or Campath-1H appears to be associated with a high incidence of CMV infections and prolonged delay of CMV immune recovery.

The early reconstitutions in the patients with RIST without ATG may be explained by early recovery of anti-CMV-specific T cells derived from recipients in a mixed chimeric state, although the majority of patients in our HSCT protocols were in a full chimeric state on day 30. Maris *et al* (2003) reported early recovery of CMV-specific T cells after low-dose total body irradiation-based RIST, possibly because of host-derived CMV-specific T cells in a mixed chimeric state. Further investigation is necessary regarding the source of CMV-specific T cells responsible for the early reconstitution of CMV immunity.

In summary, we demonstrated that the measurement of functional CMV-specific T cells with a single immunodominant CMV_{495} peptide using the IFN-γ-ELISPOT assay provided more accurate analysis of CMV immunity in HLA-A*0201 and A*0206 patients after various HSCTs than the HLA tetramer assay. RIST without ATG has an advantage of early recovery of CMV immunity compared with RIST with ATG and conventional HSCT. These results suggest that the IFN-γ-ELISPOT or HLA tetramer assay with the CMV_{495} peptide is useful for aiding decisions regarding treatment for CMV infection and prevention.

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ORIGINAL ARTICLE

Population pharmacokinetics of intravenous busulfan in patients undergoing hematopoietic stem cell transplantation

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A population pharmacokinetic analysis was performed in 30 patients who received an intravenous busulfan and cyclophosphamide regimen before hematopoietic stem cell transplantation. Each patient received 0.8 mg/kg as a 2 h infusion every 6 h for 16 doses. A total of 690 concentration measurements were analyzed using the nonlinear mixed effect model (NONMEM) program. A one-compartment model with an additive error model as an intraindividual variability including an interoccasion variability (IOV) in clearance (CL) was sufficient to describe the concentration–time profile of busulfan. Actual body weight (ABW) was found to be the determinant for CL and the volume of distribution (*V*) according to NONMEM analysis. In this limited study, the age (range 7–53 years old; median, 30 years old) had no significant effect on busulfan pharmacokinetics. For a patient weighting 60 kg, the typical CL and *V* were estimated to be 8.87 l/h and 33.8 l, respectively. The interindividual variability of CL and *V* were 13.6 and 6.3%, respectively. The IOV (6.6%) in CL was estimated to be less than the intraindividual variability. These results indicate high interpatient and intrapatient consistency of busulfan pharmacokinetics after intravenous administration, which may eliminate the requirement for pharmacokinetic monitoring.

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Keywords: intravenous; busulfan; population pharmacokinetics; NONMEM

Introduction

A high dose of busulfan in combination with cyclophosphamide is a widely used myeloablative conditioning regimen before both allogenic and autologous bone marrow transplantation (BMT).[1,2] In most cases, busulfan is administered every 6-h over four consecutive days with a total standard dose of 16 mg/kg.[1] As with most alkylating agent, busulfan has a narrow therapeutic window. The dose-limiting toxicity of busulfan in the myeloablative conditioning regimen is hepatic veno-occlusive disease (VOD), which can lead to fatal liver failure.[3,4] Following administration of the oral formulation, very wide inter- and intraindividual systemic exposure has been reported,[5] which may be linked to erratic intestinal absorption, variable hepatic metabolism, circadian rhythm, genetics, diagnosis, drug–drug interaction and age.[5–9] Recently, the intravenous formulation of busulfan has been developed in order to minimize variations of the inter- and intrainidividual systemic exposure and to provide complete dose assurance. The intravenous busulfan is registered in the USA (Busulfex™) and in Europe (Busilvex™) for adults. The recommended dosage was 0.8 mg/kg/dose for 16 consecutive doses in adults.[10–12] There have been several reports about intravenous busulfan pharmacokinetics,[10–13] with only a few applying population pharmacokinetic analysis.[13] We report here, the results of the population pharmacokinetic modeling of intravenous busulfan. The aim of this analysis was to characterize the pharmacokinetics of intravenous busulfan, including the IOV and covariate relationships in patients.

Materials and methods

Patients

A total of 30 Japanese patients (27 adults and three children) receiving a first BMT entered in a Phase 2 study were investigated. These patients received busulfan at 0.8 mg/kg as a 2 h infusion every 6 h for four consecutive days. Following busulfan therapy, patients were given cyclophosphamide at 60 mg/kg as a 3 h infusion daily for 2 days. In order to prevent seizures, phenytoin (5–10 mg/kg/day) was administered orally for 8 days, starting 2 days before the start of busulfan therapy. The following demographic and physiopathological data were considered in the analysis: diagnosis, acute myeloid leukemia (13), acute lymphocytic leukemia (5), chronic

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myelogenous leukemia (5), myelodysplastic syndrome (3), non-Hodgkin's lymphoma (4); gender, male (20), female (10); age, 7–53 years (median = 30 years); actual body weight (ABW), 18.5–82.7 kg (median = 64.1 kg); height, 111–180 cm (median = 165.5 cm); body mass index, 14.40–29.10 kg/m^2 (median = 22.65 kg/m^2); serum albumin, 3.5–4.8 g/dl (median = 4.3 g/dl); creatinine, 0.2–1.2 mg/dl (median = 0.7 mg/dl); serum alanine transaminase (ALT), 8.0–109.0 IU/l (median = 21.0 IU/l); history of hepatic disease, no (27), yes (3); concomitant antifungal treatment, no (7), yes (23); concomitant 5-HT$_3$ antiemetic treatment, no (16), yes (14). The study was approved by an independent Ethical Committee at each center. All patients provided written informed consent before enrollment.

Pharmacokinetic sampling and busulfan determination
Serial blood samples were drawn from each patient immediately before the first and ninth busulfan dose and then 0.25, 0.5, 0.75, 1.92, 2.25, 2.5, 3, 4, 5 and 6 h after the start of the first and ninth dose. The 13th dose sampling of each patient was made immediately before the infusion and at 1.92 h from the start of infusion, respectively. Plasma samples obtained by centrifugation were stored frozen until analysis. Busulfan was assayed by a validated gas chromatographic-mass selective detection (GC-MSD) assay technique.[14] The calibration curves were linear over concentrations ranging from 62.5 (quantification limit) to 2000 ng/ml. Samples with a concentration higher than 2000 ng/ml were diluted such that the concentration fell within the range of the calibration curve. Acceptance criteria for validating the analytical results of each run were as follows. Quality control (QC) samples in duplicate at three concentrations (125, 500, and 1500 ng/ml) were incorporated into each run. The results of the QC samples provided the basis for accepting or rejecting the run. At least four of six QC samples had to be within ±20% of their respective nominal values, and two of six QC samples (both at the same concentration) had also to be within the ±20% respective nominal value. The GC-MSD for pharmacokinetic investigation was performed at BML Inc. (Saitama, Japan). A total of 690 concentration measurements were available.

Population pharmacokinetic analysis and model validation
Data were analyzed using the nonlinear mixed effect model (NONMEM) program (version 5.0, Globomax LLC, Hanover, MD, USA). As the population pharmacokinetic model is used for prediction, it is important to develop a model with validation.[15] Owing to the limited number of patients in this study, external validation of the population pharmacokinetic model could not be applied; therefore, the model was evaluated using bootstrapping, one of the internal validation techniques.[15,16]

Population pharmacokinetic modeling steps were as follows: (1) a basic pharmacokinetic modeling using the NONMEM program and obtaining Bayesian individual parameter estimates, (2) validation of a basic model using the bootstrap resampling technique, (3) generalized additive modeling (GAM) for the selection of covariate candidates, (4) final pharmacokinetic modeling to determine the covariate model, and (5) validation of the final model. The NONMEM program and PREDPP package were used throughout the analysis. The first-order conditional estimation with interaction method was used in all analysis processes because of the extensive sampling design in the study. Initial pharmacokinetic parameter estimates for NONMEM modeling were calculated using the mean data obtained from all the patients by WinNonlin (version 3.3, Pharsight Corp., Mountain View, CA, USA).

Step 1: basic pharmacokinetic modeling without bootstrapping. One-compartment structural model with constant rate infusion was fitted to the busulfan concentration–time data. Interindividual variability in clearance (CL) was modeled using an exponential error model, as follows:

$$CL_i = CL \cdot \exp(\eta_i),$$

where CL_i represents the hypothetical true CL for the ith individual, CL is the typical population value of CL and η is independent, identically distributed random variables with mean 0 and variance ω^2. Interindividual variability in volume of distribution (V) was similarly modeled.

Residual intraindividual variability was identically distributed and was modeled using the additive error, constant coefficient of variation (CCV) error or the combination of the additive and CCV error models. The additive error model is described by the following equation:

$$Cp_{ij} = Cp_{mij} + \varepsilon_{ij},$$

where Cp_{ij} is the ith measured concentration in the jth individual and Cp_{mij} is the ith concentration predicted by the model at the ith observation time for the jth individual. ε is independent random variable with mean zero and variance σ^2. The magnitude of residual intraindividual variability usually depends on measurement, dosing, sampling and model misspecification errors.

IOV was introduced into the model as previously proposed.[17] The following expression was used for CL

$$CL_{ij} = CL \cdot \exp(\eta_i + \kappa_{ij}),$$

where CL_{ij} represents the hypothetical true CL for the ith individual at occasion j, CL is the typical population value of CL and η and κ are independent, identically distributed random variables both with mean 0 and variance ω^2 and π^2, respectively. IOV in V was similarly modeled.

With the fixed and random effects chosen, empirical Bayes estimates of pharmacokinetic parameters were subsequently obtained using POSTHOC option within the NONMEM program. The choice of a basic population model was based on monitoring the Akaike's information criterion (AIC). The reliability of the model selection was checked by the analysis of residual and by the visual inspection of plots of predicted versus measured concentrations.

Step 2: validation of a basic model using the bootstrap resampling technique. Resampling the original data with replacements generated 100 bootstrap samples. The resampling unit comprises samples obtained from each

patient. The appropriate structural model that best describes the data from each sample was determined. This was performed to ensure that the model, which best described the bootstrap data was not different from the basic used for developing the population pharmacokinetic model in the subsequent step. In addition, density plots of each pharmacokinetic parameter estimate were used to examine the adequacy of the basic model.

Step 3: selection of covariate candidates. Exploratory data analysis was performed on the empirical Bayesian parameter estimates from step 2 and treated as data to examine the distribution, shapes and relationships between covariates and individual pharmacokinetic parameter estimates.

The data were subjected to a stepwise (single term addition/deletion) procedure using the GAM procedure in the Xpose program (version 3.1)[18] running on the S-PLUS statistical software package (version 6.0, Insightful Corp., Seattle, WA, USA). Each covariate was allowed to enter the model in any of several functional representations. AIC was used for model selection.[19] At each step, the model was changed by the addition or deletion of the covariate that results in the largest decrease in AIC. The search was stopped when AIC reached a minimum value.

Step 4: population model building using NONMEM. For each NONMEM analysis, the improvement in fit obtained upon the addition of a covariate selected from step 3 to the regression model was assessed by changes in the NONMEM objective function. Minimization of the NONMEM objective function, equal to twice the negative log-likelihood of the data, is equivalent to maximizing the probability of the data. The change in the objective function of the NONMEM value is approximately χ^2 distributed. A difference in the NONMEM objective function value of 3.84, associated with a *P*-value of less than 0.05, was considered statistically significant.

The construction of the regression model for each structural model parameter was performed in three steps using the original data set. Covariates were first screened individually. The full model was then defined as incorporating all significant covariates. Lastly, the final model was elaborated by backward elimination from the full model.

Step 5: validation of the final population pharmacokinetic model. Two hundred bootstrap samples were generated by resampling with replacements and used for the evaluation of the stability of the final model built in step 4. The final population pharmacokinetic model was fitted repeatedly to the 200 additional bootstrap samples. The mean parameter estimates obtained from these bootstrap replications were compared with those obtained from the original data set.

The area under the plasma concentration–time curve
The area under the plasma concentration–time curve (AUC) in each patient was calculated according to the linear trapezoidal rule using WinNonlin. The AUC at the steady state was calculated for the ninth dose from dosing interval (from zero to last sampling time). The AUC in one of 30 patients after the ninth administration was not calculated because the last sample at the ninth dose was collected after the start of the next dose.

Results

Determination of a basic pharmacokinetic model
Plasma concentration versus time curves are shown in Figure 1. Parameter estimates of various structural models are given in Table 1. The models including IOV gave lower AIC values than the models not including IOV. The Additive model, including IOV and the combination of the additive and CCV error models (the combination error model) including IOV gave similar AIC values. Analysis of residuals and plots of observed versus predicted concentrations were performed to check the reliability of the basic model selection. The residuals calculated in the additive model including IOV were not obviously different from those obtained in the combination error model including IOV (data not shown). The stability of these two models was examined in a subsequent step.

Stability of the basic model as assessed using the bootstrap resampling technique
One hundred bootstrap replicates were generated from the original data and used for the evaluation of the stability of

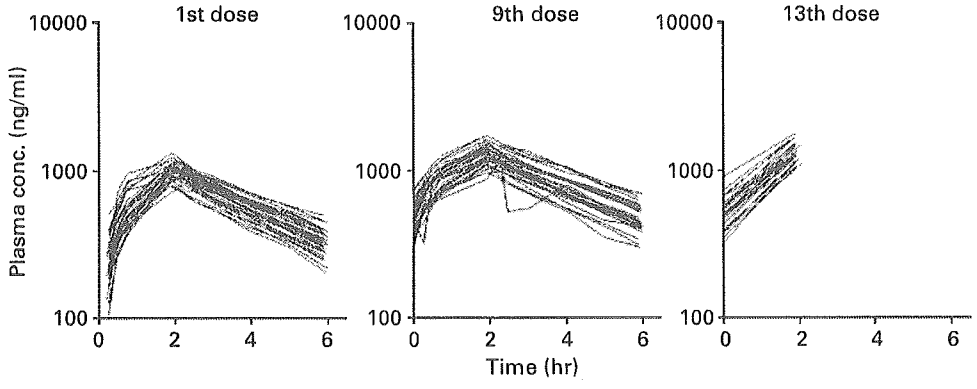

Figure 1 Observed plasma busulfan concentrations versus time.

Table 1 Parameter estimates of various models

Residual error	*IOV*	*CL (l/h)*	V *(l)*	*AIC*
CCV	No	8.56	28.9	7489
Additive	No	8.73	33.9	7235
Combination	No	8.73	33.8	7236
CCV	Yes	8.72	30.0	7421
Additive	Yes	8.77	33.4	7232
Combination	Yes	8.78	33.3	7233

IOV = interoccasion variability; CL = clearance; V = volume of distribution; AIC = akaike's information criterion; CCV = constant coefficient of variation model; additive = additive error model; combination = combination of the additive and CCV.

Table 2 Parameter estimates of the basic model

Parameter	*Estimates*
θ_{CL} (l/h)	8.77
θ_V (l)	33.4
ω_{CL} (%)	27.1
ω_V (%)	26.0
π_{CL} (%)	7.4
π_V (%)	1.4×10^{-3}
σ (ng/ml)	93.9

the basic pharmacokinetic model selected in the previous step. The parameter estimates could be obtained from all bootstrap data sets using the additive error model including IOV; however, one of 100 bootstrap data sets using the combination error model including IOV did not result in convergence. It was found that the additive model including IOV was more stable than the combination error model including IOV. Each parameter distribution of the additive error model including IOV is in a narrow range and almost unimodal (data not shown). Therefore, the additive error model including IOV was selected as the optimum basic model and was used in subsequent steps. Parameter estimates of the basic model are given in Table 2. As can be seen, the value of IOV in V is small, the decision was made whether the IOV introduces into V or not in subsequent steps. Plots of observed versus predicted concentration for the basic model are shown in Figure 2a.

Selection of covariate candidates

GAM analysis indicated that CL and V are functions of ABW (data not shown).

Population model building and stability of the final population models

The population model with covariates was built using the NONMEM program on the basis of the result of GAM analysis. ABW was found to be the predictor of both CL and V with a log-likelihood difference (LLD) of more than 10.83 ($P<0.001$) between each model in which ABW was introduced singly, and the basic model of each pharmacokinetic parameter modeled without ABW (data not shown). The full regression model was that following the allometric equations: $CL = \theta_1 \cdot (ABW/60)^{\theta 2}$, $V = \theta_3 \cdot (ABW/60)^{\theta 4}$, where θ_1 and θ_3 are the population values of CL and volume of distribution for the 60-kg patients. The IOV was not introduced into V in the population model since the IOV values obtained from each covariate model were negligible and the other parameter estimates were not changed by the introduction of IOV in V (data not shown). The full model was tested against the reduced models (Table 3).

The final population pharmacokinetic model obtained from the previous step was fitted repeatedly to the 200 bootstrapped samples. The parameter estimates of the final model using the original data and the mean parameter estimates obtained from the 200 bootstrap replicates are provided in Table 4. The mean parameter estimates were within 15% of those obtained with the original data set. Plots of observed versus predicted concentrations for the final model are shown in Figure 2b. Plots of individual parameter values obtained from the model-independent technique versus ABW are shown in Figure 3. The final

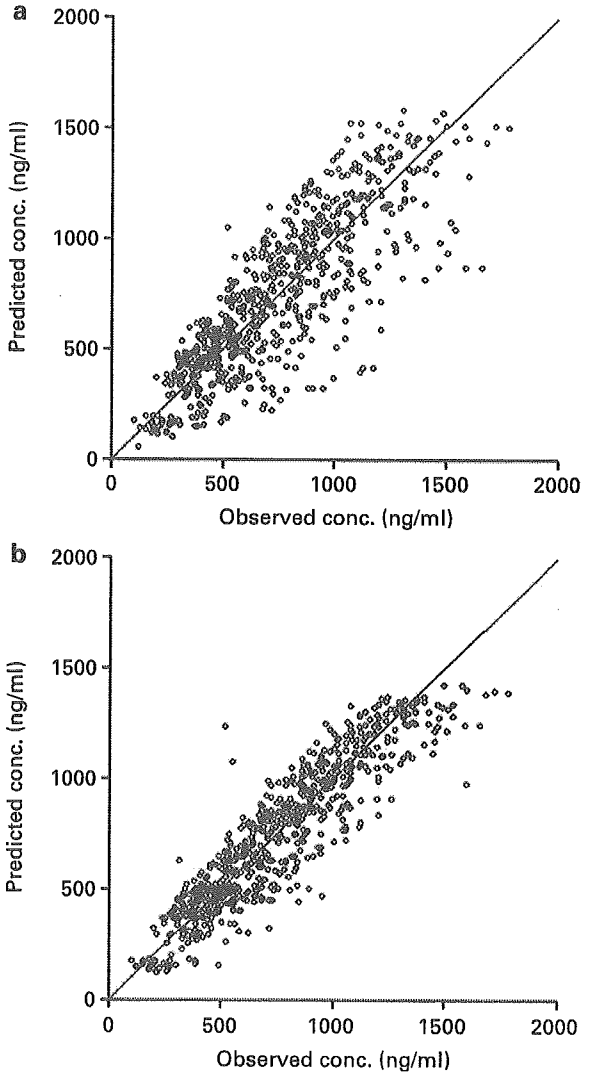

Figure 2 Plots of observed versus predicted concentration for the basic model (**a**) and for the final model (**b**).

Table 3 Comparison of the full and reduced model

Regression model	*LLD (versus full model)*
Full model	
$CL = \theta_1 \cdot (ABW/60)^{\theta 2}$	0
$V = \theta_3 \cdot (ABW/60)^{\theta 4}$	
Reduced model	
$\theta_2 = 0$	38.2*
$\theta_4 = 0$	64.9*

*$P<0.001$.
LLD = log-likelihood difference.

Table 4 Typical population parameter estimates and stability of the final model

Parameters	*Typical population parameter estimate (s.e.)*[a]	*Mean population parameter estimate (s.e.)*[b]	*Difference (%)*[c]
θ_1[d] (l/h)	8.87 (0.23)	8.86 (0.23)	−0.1
θ_2[d]	0.833 (0.077)	0.833 (0.103)	0.0
θ_3[e] (l)	33.8 (0.6)	33.8 (0.7)	0.1
θ_4[e]	0.889 (0.049)	0.889 (0.060)	0.0
ω_{CL} (%)	13.6	13.2 (1.6)	−3.6
ω_V (%)	6.3	5.6 (2.3)	−12.3
π_{CL} (%)	6.6	6.1 (2.4)	−7.9
σ (ng/ml)	94.3	94.0 (8.3)	−0.3

[a]Obtained from the original data.
[b]Mean (s.e.) calculated from 200 bootstrap replicates.
[c](Bootstrap mean value-typical value from final model)/bootstrap mean value × 100(%).
[d]$CL = \theta_1 \cdot (ABW/60)^{\theta 2}$.
[e]$V = \theta_3 \cdot (ABW/60)^{\theta 4}$.

population model was well described the relationships between the pharmacokinetic parameters and ABW.

Discussion

The objective of population pharmacokinetic analysis was to characterize the pharmacokinetics of the intravenous busulfan including IOV and covariate relationships in patients. Reliability of results obtained from population analyses depends on the modeling procedure. Therefore, the evaluation of basic (covariate-free model) and final (covariate model) population pharmacokinetic models was performed using bootstrap resampling because of the limited number of patients in the study.

The one-compartment model with an additive error model including IOV in CL was selected as the population model during model development. The final population pharmacokinetic model built in the study was fitted to the 200 bootstrap samples. The mean parameter estimates obtained with the 200 bootstrap replicates of the data were within 15% of those obtained from original data. This indicates that the final model is stable.

With regard to the effect of covariates investigated in this analysis on the pharmacokinetic parameters of busulfan after intravenous infusion, the ABW was found to be a

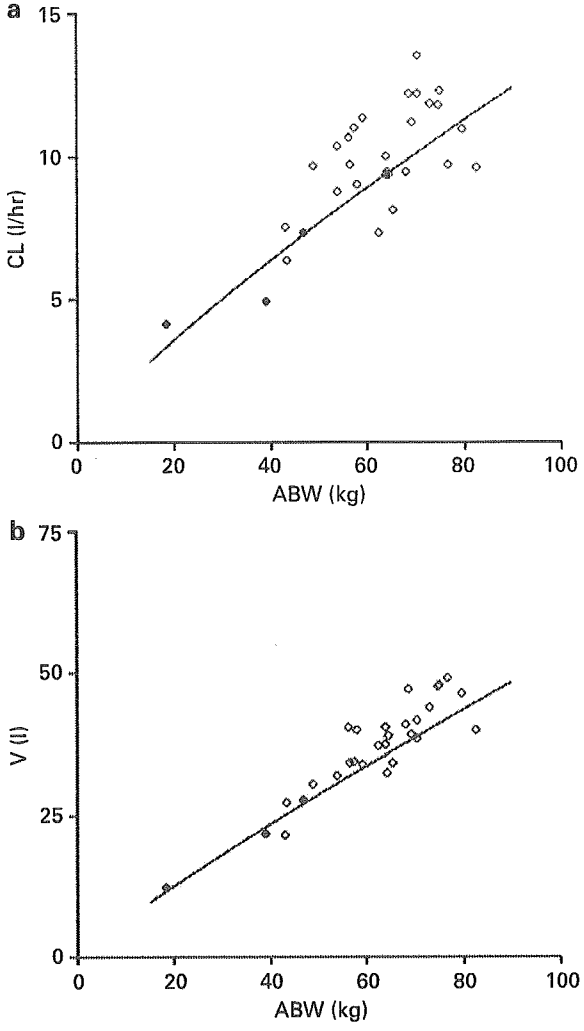

Figure 3 Plots of individual parameter values versus actual body weight. ABW, actual body weight. Clearance (CL) (**a**) and the volume of distribution (V) (**b**) after the first administration were calculated according to noncompartmental analysis using WinNonlin. Lines represent the estimates predicted the proposed allometric equations. Open and closed circles represent the value in adults and children, respectively.

determinant of CL and V. In the previous studies, age, ABW, body surface area (BSA), ALT and concomitant phenytoin treatment were reported as possible covariates of oral busulfan pharmacokinetics.[5–9,20,21] After the intravenous administration of busulfan, the relationships between ABW and pharmacokinetic parameters were reported.[13] Since physiological function was relatively well controlled in our study, variation of covariates was in a narrow range or within the normal limits. The limitation of developing population models based on such a small, relatively uniform patient population has been reported.[22] Therefore, the relationships between covariates and the pharmacokinetic parameters of intravenous busulfan need further investigation in a larger population, especially in younger children.

In general, a nomogram based on the population approach is a useful tool for dose adjustment, and therapeutic drug monitoring (TDM) is another powerful

tool. With TDM, some of the unknown interindividual variations can be quantified and total fluctuations in drug exposure can be reduced after dose adjustment. There are several reports about TDM techniques of oral busulfan.[20,23,26]

To compare our data with the oral busulfan studies in adult patients, we used previously published data.[23] The selected study has similar sampling points to our study and individual AUC were reported. Several reports indicate that an AUC of 900–1500 μmol min/l[4,6,24,25] in patients receiving a conventional busulfan regimen prevents treatment failure and the risk of fatal toxicities. In the previous study, the observed AUC at a steady state in 8/12 (66.7%) patients without dose adjustment fell in the above range. The percentage of patients within the range increased to 92.9% (13/14) with the dose adjustment according to the TDM results. In our study, the observed AUCs at steady state in 25/29 (86.2%) patients were within the range.

TDM requires blood sampling, drug concentration measurement and pharmacokinetic analysis. In the case of TDM after the administration of busulfan, it takes 1 or 2 days to adjust the dosage regimen using TDM data. Therefore, 25–50% of busulfan exposure remains uncontrollable in the standard regimen of 16 doses four times per day for 4 days. The contribution of the TDM for busulfan therapy is limited because of the reason described above. Intravenous busulfan may have an advantage over the oral busulfan in the limited term therapy since the systemic exposure of intravenous busulfan is expected to be reproducible throughout the treatment as compared with that of oral busulfan with TDM.

Oral busulfan is generally used at a dose of 1 mg/kg in adults, but the recommended dosage of intravenous busulfan is 0.8 mg/kg.[10–12] The previous report suggests that 1 mg/kg oral busulfan is a slight over-dosage.[27] According to the previous report, the benefit of the intravenous busulfan administration is not only the decrease in the variability of systemic exposure (AUC), but also the optimizing of average exposure during busulfan dosing.[28] In order to come to a definite conclusion of the clinical benefit of the intravenous busulfan, a prospective comparison of exposure of intravenous versus oral administration might be needed.

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