Patients and methods

We surveyed transplantation centers in Japan and identified three centers (Komagome Hospital, Kyushu University and National Cancer Center Hospital) that were performing a prospective clinical trial against various advanced solid tumors including pancreatic cancer. The University of Tokyo Hospital was performing a trial that exclusively included patients with advanced pancreatic cancer. Two of these trials have already been published.[14,15] We collected the clinical results of all patients with pancreatic cancer who participated in these studies from the published papers or using a questionnaire.

The reduced-intensity conditioning regimens were exclusively fludarabine-based, but varied among centers. The most intensive regimen was the combination of fludarabine (30 mg/m^2/day for 6 days), BU (4 mg/kg/day for 2 days) and gemcitabine (1000 mg/m^2/day for 3 days) at the University of Tokyo Hospital, whereas the combination of fludarabine (30 mg/m^2/day for 3 days) and TBI at 2 Gy (Kyushu University) was the least intensive. CY (60 mg/kg/day for 2 days) was combined with fludarabine (25 mg/m^2/day for 5 days) in the Komagome Hospital. Prophylaxis against GVHD was performed with CYA either alone or in combination with MTX or mycophenolate mofetil. PBSCs were mobilized with G-CSF, cryopreserved using standard techniques without *ex vivo* manipulation, thawed and infused on day 0. Host/donor T-cell chimerism was analyzed by sex-chromosome FISH or the short tandem repeat method after transplantation.[16]

The tumor response to treatment was evaluated as described previously.[15] Briefly, CR (complete response) was defined as disappearance of all clinical evidence of tumor for a minimum of 4 weeks by computed tomography scan. MR (minor response) and PR (partial response) were defined as decreases of 25–50% and greater than 50%, respectively, in the sum of the products of the maximum diameter and its perpendicular diameter of all measurable lesions for a minimum of 4 weeks.[7]

Engraftment was defined as a neutrophil count more than 500/mm^3 for 3 consecutive days after RICT. Engraftment failure was diagnosed as when engraftment was not achieved at any time after transplantation. The probability of survival was calculated using the Kaplan–Meier method. The incidence of chronic GVHD was evaluated in 13 patients who survived longer than 100 days after RICT. Univariate comparisons for dichotomous and time-to-event variables between groups were performed with the Fisher exact test and the log-rank test, respectively, and multivariate analyses were performed using logistic regression analysis and proportional hazards modeling, respectively. Factors associated with at least borderline significance ($P<0.10$) in the univariate analysis were subjected to a multivariate analysis using backward stepwise selection of covariates. All P-values were two sided and values of 0.05 or less were considered statistically significant.

Results

Clinical data of 22 patients with a median age of 57 years (range: 36–68 years) were collected (Table 1). There were 15 male and seven female patients. Fifteen patients had metastatic disease, whereas 7 had locally advanced diseases. All but one patient had received chemotherapy with gemcitabine either alone or in combination with other antineoplastic agents before RICT. In all, 10 had received local irradiation in addition to chemotherapy. Eastern Cooperative Oncology Group performance status (ECOG-PS) was equal to or greater than 2 in 10 patients. The conditioning regimen was fludarabine-BU-based in 10, fludarabine-CY in 7 and fludarabine-TBI in 5. The donors were HLA-matched relatives except in one patient who received graft from an HLA-mismatched family donor. The number of CD34-positive cells infused was greater than 4.0×10^6 cells/recipient body weight (kg) in 10 patients. CYA was used for GVHD prophylaxis: alone in 8, combined with MTX in 10 and combined with mycophenolate mofetil in 4.

Engraftment was observed in all but two patients with a median duration from RICT of 12 days (range: 6–42 days). Complete donor-type T-cell chimerism was confirmed in 18 patients, whereas mixed chimerism persisted in 4 patients. A total of 12 patients developed grade II–IV acute GVHD. Limited and extensive chronic GVHD was observed in three and five patients, respectively, among the 13 patients who survived longer than 100 days after RICT.

The best response after RICT was CR in one, PR in two, MR in two and stable disease in eight. The overall response

Table 1 Characteristics of the patients

Age (years)	
Median	57
Range	36–68
Sex	
Male	15
Female	7
Disease	
Locally advanced	7
Metastatic	15
ECOG-PS	
0–1	12
2–4	10
Regimen	
Flu + BU + Gem	7
Flu + CY	6
Flu + TBI	6
Flu + BU	3
Donor	
HLA-matched sibling	21
Mismatched family donor	1
CD34+ cells in graft	
$\leq 4.0 \times 10^6$/kg	12
$> 4.0 \times 10^6$/kg	10
GVHD prophylaxis	
CsA alone	8
CsA + MTX	10
CsA + MMF	4

Abbreviations: ECOG-PS = Eastern Cooperative Oncology Group performance status; Flu = fludarabine; Gem = gemcitabine; MMF = mycophenolate mofetil.

rate (CR + PR + MR) was 23%. A univariate analysis to identify possible relationships between clinical parameters and overall response failed to show any statistically significant factors. The conditioning regimen did not significantly affect the response rate, although the statistical power was not enough due to the small number of patients in each group. Response was observed in two of the seven patients who received the most intensive regimen including fludarabine, BU and gemcitabine, while it was seen in one of the six patients who received the least intensive regimen with fludarabine and low-dose TBI. None of the patients with mixed chimerism showed a response, but this difference was not statistically significant. DLI (donor lymphocyte infusion) was performed in four patients who had progressive disease after RICT, and the number of infused CD3-positive cells was between 2.7×10^7 and 1.8×10^8 cells/kg. One patient showed tumor shrinkage after DLI, but the response was transient.

Figure 1a shows overall survival after RICT. Median survival was only 139 days and the major cause of death was tumor progression. Other causes of death included infection in one and chronic GVHD in two. In a univariate analysis, ECOG-PS below 2 and infused CD34-positive cell dose greater than 4.0×10^6 cells/kg were associated with significantly longer survival after RICT (Table 2; Figures 1b and c). A multivariate analysis revealed that these two factors were almost independently significant (Table 2). With regard to post transplantation factors, while the development of grade II–IV acute GVHD did not significantly affect survival ($P = 0.76$), the eight patients who developed chronic GVHD tended to survive longer than those who survived longer than 100 days after RICT but did not develop chronic GVHD ($P = 0.092$; Figure 2). This analysis was unlikely to be biased by the fact that patients who survived longer had more chance to develop chronic GVHD, as most of the patients developed chronic GVHD as a progressive type from acute GVHD.

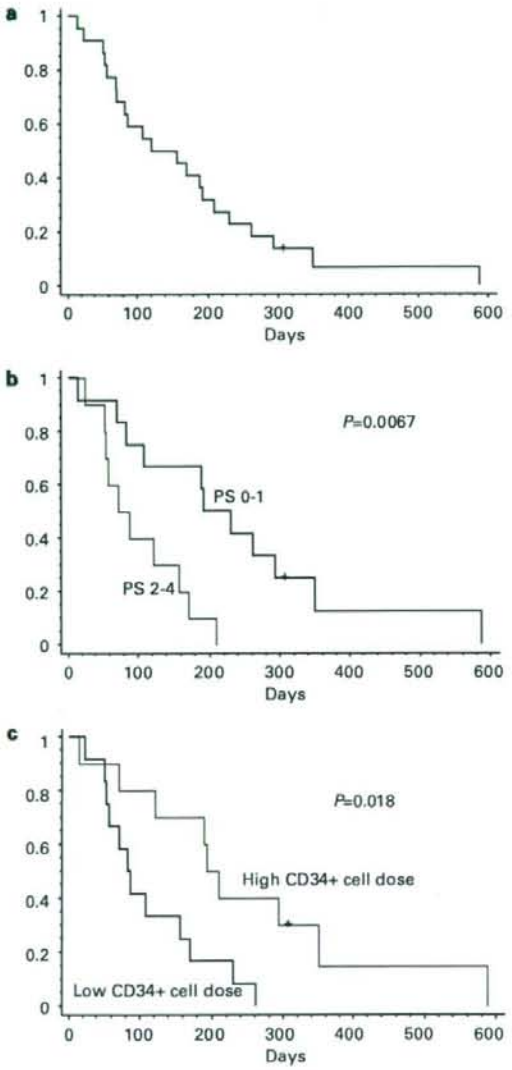

Figure 1 Patient survival, overall (**a**) and grouped according to risk factors (**b** and **c**).

Discussion

To summarize these findings, 23% of the 22 patients in this series showed a response to RICT. However, the duration of the response was generally short and most of the patients eventually died with progressive disease. The median survival after RICT was only 139 days and only one survived longer than 1 year after transplantation. Good ECOG-PS and higher number of CD34-positive cells in the graft were independently associated with longer survival.

The relationship between the number of infused CD34-positive cells and transplant outcome has been studied in PBSC transplantation for hematological malignancies.[17] The infusion of a higher number of CD34-positive cells has been associated with faster recovery of neutrophils and plts, but chronic GVHD was more frequently observed in patients who received a very high dose of CD34-positive cells (that is, $> 8.0 \times 10^6$ cells/kg). In this study, two patients failed to achieve engraftment, and both had received less than 4.0×10^6 cells/kg of CD34-positive cells. However, a statistically significant survival advantage was confirmed even after these two patients were excluded from the analysis. If we consider that the major cause of death in this study was progressive disease, the infusion of a higher number of CD34-positive cells might have protected patients from disease progression by a graft-versus-host reaction, although we failed to show a significant difference between the number of infused CD34-positive cells and tumor response or the incidence of chronic GVHD, probably due to the small number of patients. Patients who developed chronic GVHD showed better survival than those who did not, with a borderline significance, suggesting that they had some immunological protection against the progression of pancreatic cancer.

Table 2 Univariate and multivariate analyses for overall survival

Factor	*Median survival (days)*	P-*value*
A. Univariate		
Age (years)		
< 55	115	0.63
⩾ 55	180	
Sex		
Male	87	0.69
Female	170	
ECOG-PS		
0–1	211	0.0067
2–4	80	
Stage		
Locally advanced	192	0.21
Metastatic	121	
Serum CEA		
Negative	122	0.70
Positive	192	
Serum CA19-9		
Negative	157	0.84
Positive	132	
Regimen		
Flu + BU based	191	0.25
Flu + CY	156	
Flu + TBI	71	
CD34+ cell dose		
$\leqslant 4.0 \times 10^6$/kg	85	0.018
$> 4.0 \times 10^6$/kg	201	
GVHD prophylaxis		
CsA alone	132	0.55
CsA + MTX	191	
CsA + MMF	96	
B. Multivariate	*Relative risk (95% CI)*	P-*value*
ECOG-PS		
0–1	1.00	0.032
2–4	3.39 (1.11–10.3)	
CD34+ cell dose		
$\leqslant 4.0 \times 10^6$/kg	1.00	0.068
$> 4.0 \times 10^6$/kg	0.37 (0.13–1.07)	

Abbreviations: CI = confidence interval; ECOG-PS = Eastern Cooperative Oncology Group performance status; Flu = fludarabine; MMF = mycophenolate mofetil.

This study was limited by the heterogeneity of transplantation procedures among centers. However, considering the difficulty of performing a large-scale prospective study on RICT against pancreatic cancer, this small survey may currently represent the best evidence of the efficacy of this novel treatment strategy against advanced pancreatic cancer and may suggest a future direction for improving the treatment outcome. We showed that pancreatic cancer can be a possible target for allogeneic immunotherapy. However, the immunological effect was not strong or durable enough to prevent tumor progression. A possible strategy for enhancing a graft-versus-tumor effect against pancreatic cancer without enhancing GVHD is a combination with specific immunotherapy using antigens including CA19-9,

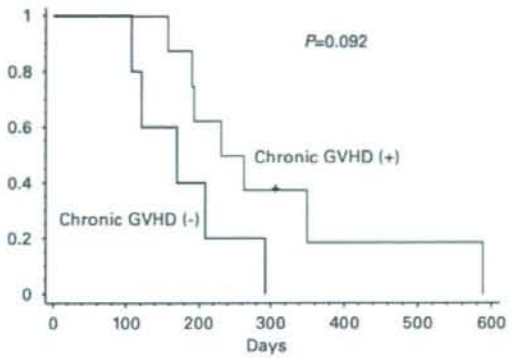

Figure 2 Overall survival of patients who survived at least 100 days after transplantation grouped according to the presence or absence of chronic GVHD.

CA242, CEA, Her-2, mutated K-ras and MUC-1.[12] Among these, CEA is attractive, since it is expressed in 85–90% of pancreatic cancer, and a specific immunotherapy against CEA could also be applied to other gastrointestinal cancers. An increase in the serum anti-CEA antibody level associated with a tumor response was observed in the University of Tokyo Study.[15] In addition, Kim *et al.*[18] showed that a peptide CEA652, TYACFVSNL, binds to HLA-A24 and induces CEA-specific cytotoxic T cells. Therefore, vaccination with such a peptide may be promising as a post transplantation immunotherapy against pancreatic cancer. Another approach is to add molecular targeting agents such as erlotinib after RICT. This may induce tumor cell death, leading to the enhanced presentation of tumor antigens to donor T cells. In addition, RICT can be combined with surgical resection, since the prognosis of pancreatic cancer is very poor even after complete resection.[19,20] Maximum graft-versus-tumor effect can be expected when the tumor load is at its lowest level.

In conclusion, a tumor response was observed in approximately one-fourth of the patients who underwent RICT against advanced pancreatic cancer. Although the response was not durable, our findings, such as the relationship between longer survival and the infusion of a higher number of CD34-positive cells or the development of chronic GVHD, should support a future study to enhance the specific immunological effect against pancreatic cancer.

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ORIGINAL ARTICLE

Stromal cells in bone marrow play important roles in pro-inflammatory cytokine secretion causing fever following bortezomib administration in patients with multiple myeloma

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Abstract Bortezomib blocks the activation of nuclear factor-κB-mediated pro-inflammatory cytokines, however, systemic inflammatory symptoms following bortezomib administration have been reported, although their mechanisms remain elusive. Serum samples were obtained from five patients, who participated in a phase I/II study of Japanese patients with relapsed or refractory multiple myeloma (MM), and developed cyclic fever following bortezomib administration, to measure cytokine levels. Significant correlations between interleukin (IL)-6 or interferon (IFN)-γ and the body temperature were observed in two patients each. Furthermore, we found that IL-6 elevation was not observed after the addition of bortezomib to any examined MM cells alone, but was noted in a case of bone marrow stromal cells (BMSCs) of macrophage origin alone or co-cultured with MM cells. Similarly, a marked increase in IFN-γ levels was induced by adding bortezomib to BMSCs of fibroblast origin. Although this investigation was a preliminary study with a small number of patients, our results suggested that pro-inflammatory cytokines causing bortezomib-associated fever were secreted from BMSCs rather than MM cells.

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Keywords Multiple myeloma · Bortezomib · Bone marrow stromal cells · Pro-inflammatory cytokine · IL-6 · IFN-γ

1 Introduction

Multiple myeloma (MM) is one of the incurable hematological malignancies that continues to relapse or progresses even with conventional treatment modalities. Since conventional-dose melphalan-predonisone chemotherapy and high-dose therapy of melphalan were established, there have been no available treatment options with durable efficacy for patients with relapsed or refractory MM for the past several decades, and so more efficacious therapies are strongly desired. The first of the class of proteasome inhibitors, bortezomib, is a novel agent selectively targeting the 20S proteasome, which exhibits an anti-myeloma effect in part by the inactivation of nuclear factor (NF)-κB-mediated pro-inflammatory cytokines [1], and, therefore, would be expected to have anti-inflammatory effects [2]. Indeed, promising results using bortezomib have already been reported not only in patients with relapsed or refractory MM [3–5], but also in those with previously untreated MM [6].

On the other hand, there are some reports of systemic inflammatory symptoms including lung injury [7–9] and fever [3, 7, 10] following bortezomib administration, which were not associated with infection, although their mechanisms remain elusive. Although the incidence of fever following bortezomib administration was around 20% [3, 4], fever is one of the adverse events which cannot be ignored because it has a marked influence on the patient's activities of daily living. Moreover, it might lead to the development of lung injury, which sometimes results in a

fatal outcome [7–9]. Therefore, clarification of the mechanism of fever following bortezomib administration is important in order for clinicians who treat patients with proteasome inhibitors for MM to understand the contradictory inflammatory syndromes after bortezomib administration.

In the present study, we examined the relationship between serum cytokines and fever following bortezomib administration in patients with MM using statistical analyses, and elucidated the exact mechanism of fever employing cell lines derived from MM and/or bone marrow stromal cells (BMSCs).

2 Materials and methods

2.1 Clinical data collection

We hypothesized that fever after bortezomib administration was associated with increased serum cytokine levels. To investigate this, we analyzed several serum cytokines such as interleukin (IL)-6, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, the clinical symptoms, and their relationship. Ten patients were enrolled from our institution in a multicenter phase I/II study of bortezomib involving 34 Japanese patients in total with relapsed or refractory MM. Bortezomib of 0.7 mg/m^2 ($n = 2$), 1.0 mg/m^2 ($n = 2$), and 1.3 mg/m^2 ($n = 6$) was administered by bolus intravenous injection on days 1, 4, 8, and 11 every 3 weeks. Serum samples were collected before each predose of cycle 2, and 12–24 h after each administration from five patients who developed fever following bortezomib administration in cycle 1 and gave written informed consent. Three (patients 6, 9, and 10) of the remaining five patients did not develop fever, and the other two patients (patients 7 and 8) did not consent to blood sampling. Serum levels of IL-6, IFN-γ, and TNF-α were examined by chemiluminescent enzyme immunoassay, enzyme-linked immunosorbent assay (ELISA), and enzyme immunoassay, respectively. We defined fever following bortezomib administration as a transient fever $\geq$37.5°C, measured on axillary body surfaces, which occurred within 36 h after administration. The responses were determined according to European Group for Blood and Marrow Transplantation criteria [11].

2.2 Cell lines and reagents

As for human MM cell lines, MM.1S was kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL, USA), and U266 and RPMI8226 were purchased from the American Type Culture Collection (Manassas, VA, USA). All MM cells were seeded at a density of 1×10^5 cells/well in 24-well plates (Iwaki, Tokyo, Japan) and cultured in RPMI1640 (Sigma Chemicals, St Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Bioserum, VIC, Australia). After MM cells were incubated with 5 nmol/l bortezomib (Millennium Pharmaceuticals, Inc., Cambridge, MA, USA) for 16 h, the plates were centrifuged at 1,500 rev/min for 5 min to collect the supernatant. As for the various cloned stromal cells established from human bone marrow, LP101, HAS303, and AA101 [12, 13] were seeded at a density of 5×10^4 cells/well in 24-well plates and cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, NY, USA) containing 10% FBS, and incubated with bortezomib for 48 h. Before co-culture with BMSCs, the culture medium of MM cells, RPMI1640, was replaced with IMDM containing 10% FBS and cultured for 2 weeks. Then, the culture medium of BMSCs was discarded and BMSCs were co-cultured with MM cells. After co-culture for 24 h, BMSCs and MM cells were further incubated with 5 nmol/l bortezomib for 16 h.

2.3 Cytokine analyses of supernatants

Interleukin-6 was measured using a Human Interleukin-6 EASIA™ ELISA kit (BioSource International, Inc., Camarillo, CA, USA), and IFN-γ and TNF-α were measured using the Cytometric Bead Array System (BD Biosciences, San Jose, CA, USA).

2.4 Statistical analyses

Wilcoxon's signed rank test and Student's t-test were used to analyze patient data and values of cytokines in cultured cells before and after bortezomib administration in statistical comparisons, respectively. Spearman's rank correlation test was used to examine correlations between serum cytokines and body temperature (BT). All statistical analyses were performed with SPSS version 11.0J. $P < 0.05$ was considered significant.

3 Results

3.1 Patient characteristics and analyses of serum cytokine levels

The details of the ten patients who were treated at our institution are summarized in Table 1. The median age was 64 years (range 35–73) with six male and four female patients. Seven of the ten patients developed fever after bortezomib administration: neither of the two patients in the 0.7 mg/m^2 cohort, both in the 1.0 mg/m^2, and five of six in the 1.3 mg/m^2. The maximum grade of fever was grade 2 according to the National Cancer Institute

Table 1 Patient characteristics

Patient number	Gender	Age (y)	Days from disease onset to bortezomib administration	Prior treatment	Type of myeloma	Stage (D–S)	Dosage of bortezomib (mg/m^2)	Fever[b] (grade)	Cytokines correlated with fever	Response to bortezomib
1[a]	F	35	593	VAD, COP–MP	IgA-κ	IIA	1.0	Yes (2)	IL-6	NC
2[a]	M	68	1,375	COP–MP	IgG-κ	IIA	1.3	Yes (2)	IL-6[c]	NC
3[a]	M	73	878	COP–MP	IgA-κ	IIIA	1.3	Yes (2)	IFN-γ	PR
4[a]	M	62	418	MP	IgG-κ	IIIA	1.3	Yes (2)	IFN-γ	CR
5[a]	M	67	615	VAD	IgA-κ	IIIA	1.3	Yes (2)	IL-6	NC
6	M	54	3,022	VAD, VMCP, MP, Thalidomide	IgG-κ	IIIA	1.3	No	ND	NC
7	F	57	1,412	ROAD, Thalidomide, VAD, HD-CPA, Melphalan/ASCT, Thalidomide	IgA-λ	IIIA	1.3	Yes (1)	ND	nCR
8	F	56	1,516	ROAD–IN, MCNU–MP, MCNU–VMP, VAD, Thalidomide–DEX	IgG-κ	IIIA	1.0	Yes (1)	ND	NE
9	M	66	480	VAD	IgA-κ	IIA	0.7	No	ND	PD
10	F	69	1,666	COP–MP, Navelbine, COP–MP	IgG-κ	IIIA	0.7	No	ND	NC

y Years, *D–S* Durie-Salmon, *M* male, *F* female, *VAD* vincristine, doxorubicin, and dexamethasone, *COP* cyclophosphamide, vincristine, and prednisolone, *MP* melphalan and prednisolone, *VMCP* vincristine, melphalan, cyclophosphamide, and prednisolone, *ROAD* ranimustine, vincristine, melphalan, and dexamethasone, *HD-CPA* high-dose cyclophosphamide, *ASCT* autologous stem cell transplantation, *ROAD-IN* ROAD and interferon, *MCNU* ranimustine, *VMP* vincristine, melphalan, and prednisolone, *DEX* dexamethasone, *ND* not done, *NC* no change, *PR* partial response, *CR* complete response, *nCR* immunofixation-positive CR (near CR), *NE* not evaluable, *PD* progressive disease

[a] Serum samples were obtained from patients who gave written informed consent to participate in this study

[b] Fever following bortezomib administration was defined as a transient fever (at least 37.5°C axillary temperature) which occurred within 36 h after drug administration and was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0

[c] Patient number 2 showed a tendency toward a correlation between serum IL-6 and fever

Common Toxicity Criteria Version 2.0 in all patients who developed fever following bortezomib administration. Three of the seven patients who developed fever achieved objective responses (one each of complete response, immunofixation-positive complete response, and partial response). In contrast, none of the three patients (two received bortezomib at a dose of 0.7 mg/m^2 and one at 1.3 mg/m^2) without fever had objective responses (Table 1).

Recurrent fever with each injection started from day 2 in three (patients 1, 2, and 3), from day 5 in one (patient 5), and from day 9 in one (patient 4) of five examined patients. BT ($P < 0.01$) and levels of IL-6 ($P < 0.01$) and IFN-γ ($P < 0.01$) in their serum significantly increased after bortezomib administration (Fig. 1a–c), whereas no one showed significant changes in their serum levels of TNF-α (data not shown). Elevations in their levels of serum IL-6 and IFN-γ following bortezomib administration were transient. Significant correlations were observed between serum IL-6 levels and BT in two patients (patient 1, $r = 0.9$, $P < 0.01$; patient 5, $r = 0.87$, $P < 0.01$), and between serum IFN-γ levels and BT in another two patients (patient 3, $r = 0.7$, $P = 0.05$; patient 4, $r = 0.76$, $P = 0.03$). Another patient showed a tendency toward a correlation between serum IL-6 levels and BT (patient 2, $r = 0.67$, $P = 0.07$) (Table 1). We analyzed serum C-reactive protein (CRP) levels as well as serum cytokines (IL-6, TNF-α, and IFN-γ) in the five patients who developed fever following bortezomib administration. Serum CRP levels significantly increased after bortezomib administration ($P = 0.04$), whereas no significant correlation was observed between serum CRP and serum IL-6 levels (data not shown). In the five patients, objective responses (one each of complete and partial responses) to bortezomib were observed only in patients who demonstrated an elevation of serum IFN-γ levels (Table 1).

3.2 Mechanism of cytokine elevation

Although no elevation of IL-6 levels in the supernatant was observed after the addition of bortezomib to MM cells alone (Fig. 2a), bortezomib increased IL-6 levels in the supernatant of LP101 cells, which originated from macrophages, when co-cultured with MM cells except for U266 (Fig. 2b) or without MM cells (Fig. 2c). Similarly, a marked elevation of IFN-γ levels was induced by adding bortezomib to AA101, which was originated from fibroblasts, without MM cells (Fig. 2d); however, bortezomib did not induce IL-6 secretion from AA101 cells either alone (Fig. 2c) or after co-culture with MM cells (data not shown). No significant elevation of IFN-γ levels in the supernatant was observed after the addition of bortezomib to MM.1S cells alone (data not shown). Bortezomib did not increase IFN-γ levels in the supernatant of U266 or RPMI8226 cells either. In the same combination between MM cells and BMSCs as used in the experiments shown in Fig. 2b, no elevation of IFN-γ levels in the supernatant was observed in any of the examined combinations after the addition of bortezomib (Fig. 2e). Although bortezomib did not increase IL-6 levels in the supernatant of AA101 cells, which originated from fibroblasts, when co-cultured with MM cells (Fig. 2f), an elevation of IFN-γ levels was induced by adding bortezomib to AA101 only when co-cultured with MM.1S (Fig. 2g). Elevation of TNF-α was not observed in any combination between MM cells and BMSCs examined (data not shown). After bortezomib administration, HAS303 cells, which were of endothelial cell origin, either with or without MM cells, did not induce IL-6 (Fig. 2c) or IFN-γ (Fig. 2d) elevation.

Furthermore, we assumed that some substance in the supernatant of MM cell culture media can induce IL-6 secretion, and the elevation of IL-6 levels was observed when only the supernatant obtained from the culture medium of MM.1S cells after bortezomib administration was added to LP101 without MM.1S cells (Fig. 2h).

4 Discussion

Because bortezomib has been shown to inhibit interactions between myeloma and stromal cells by interfering with the NF-κB-dependent induction of pro-inflammatory cytokine

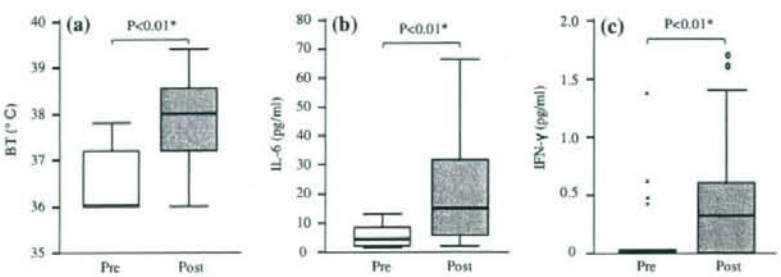

Fig. 1 Analyses of body temperature (BT) and serum cytokine levels. In five patients, their BT (**a**) and serum levels of IL-6 (**b**), and IFN-γ (**c**) significantly increased after bortezomib administration

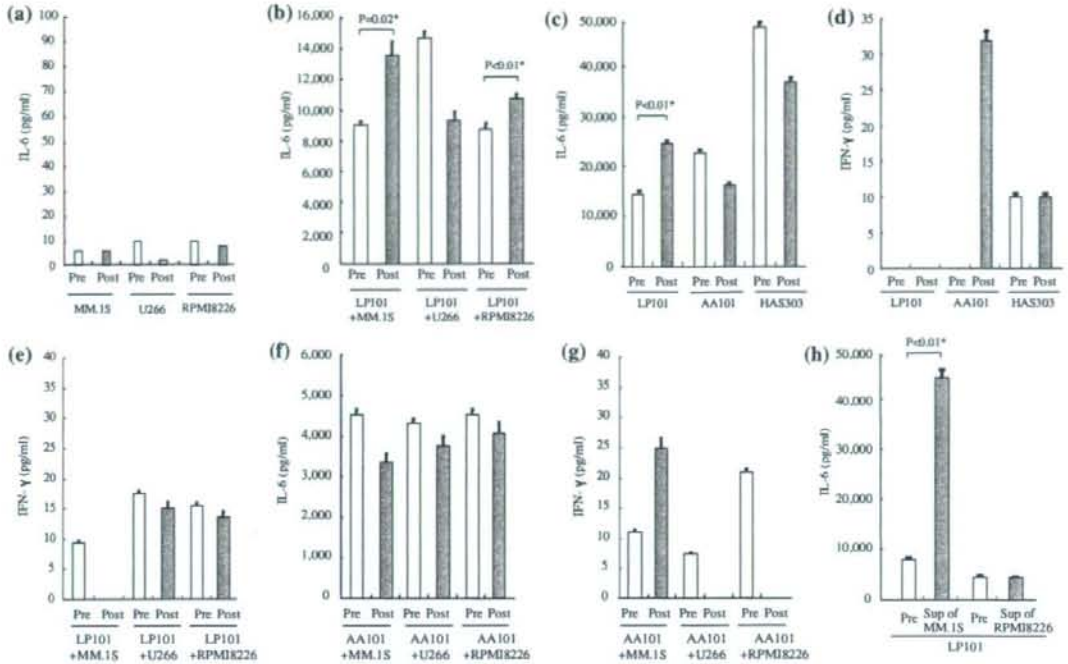

Fig. 2 Levels of cytokines in various combinations between multiple myeloma (MM) and bone marrow stromal cells. The elevation of IL-6 levels in the supernatant was not observed after the addition of bortezomib to any examined MM cells alone (**a**). Bortezomib augmented IL-6 secretion from LP101 cells when co-cultured with MM cells except for U266 (**b**). Bortezomib increased the level of IL-6 secretion from LP101 cells without MM cells (**c**). A marked elevation of IFN-γ levels was induced by adding bortezomib to AA101 cells in the absence of MM cells; however, IFN-γ was not detected at all either before or after bortezomib administration in the supernatant of LP101 cells, and the levels of IFN-γ were not changed after the addition of bortezomib to HAS303 cells (**d**). In the same combination as shown in Fig. 2b, no elevation of IFN-γ levels in the supernatant was observed after the addition of bortezomib (**e**). Although bortezomib did not increase IL-6 levels in the supernatant of AA101 cells, which originated from fibroblasts, when co-cultured with MM cells (**f**), the elevation of IFN-γ levels was induced by adding bortezomib to AA101 after co-culture with MM.1S (**g**). A marked increase in IL-6 levels was observed even when only the supernatant of the MM.1S culture medium after incubation with bortezomib was added to LP101 but not RPMI8226 (**h**). All experiments were performed in triplicate, and *error bars* indicate the mean value ± SD. *P*-values were considered significant when <0.05. Only significant *P*-values are presented in figures. *Pre* and *Post* The values before and after bortezomib administration, respectively, *Sup* the supernatant obtained from the culture medium of MM.1S or RPMI8226 cells after incubation with bortezomib

secretion from MM cells and exerting an antiangiogenic activity [1], bortezomib is believed to have anti-inflammatory effects [2]. On the contrary, our clinical observations suggested that the BT and serum levels of IL-6 and IFN-γ significantly increased after bortezomib administration, and fever following bortezomib administration was correlated with the increase in levels of serum IL-6 or IFN-γ. Therefore, we postulated that these cytokines causing the bortezomib-associated fever were released from BMSCs rather than MM cells themselves. Indeed, we found that pro-inflammatory cytokines, such as IL-6 and IFN-γ, were secreted from BMSCs, especially macrophages and fibroblasts, respectively, after incubation with bortezomib in vitro.

Some previous reports described that no significant changes [14] or decreases [15] in levels of serum IL-6 were observed after bortezomib administration in patients with various hematological diseases including MM. This discrepancy between these previous reports and our results, in which serum levels of these cytokines increased after bortezomib administration, might be attributable to differences in the timing of serum sample collection. Serum samples were collected before and 12–24 h after bortezomib administration in our study, however, those were collected before and several cycles after administration in the previous study [14]. Furthermore, there were some reports concerning the increase in levels of serum IL-6, which might be associated with inflammatory symptoms

such as lung injury or skin rash [9, 16], although the number of patients was small. These observations are partially consistent with our results.

Our results suggested that pro-inflammatory cytokines were secreted from BMSCs due to the direct effects of bortezomib on BMSCs. On the contrary, Hideshima et al. described that bortezomib decreased IL-6 levels in the supernatant of BMSCs, which were obtained from bone marrow specimens of patients with MM, when co-cultured with or without MM.1S [1]. This discrepancy might result from differences in BMSCs examined in the two studies. The BMSCs used in the previous report were the bulk of BMSCs obtained from patients with MM [1]. On the other hand, each of the BMSCs we used was well-characterized and originated from macrophages, fibroblasts, or endothelial cells [12, 13]. Our results suggested that macrophages played a role in cytokine secretion specific to IL-6 and fibroblasts did specific to IFN-γ after bortezomib administration. The quantity of IL-6 mRNA in LP101 cells before bortezomib administration and after incubation with bortezomib for 48 h was analyzed using the StepOnePlus™ Real Time PCR System (Applied Biosystems, Tokyo, Japan). The cycle threshold values after were lower than those before bortezomib administration (data not shown). This result of real-time PCR was consistent with our experimental results regarding elevated IL-6 following bortezomib administration measured using the ELISA kit.

We further examined whether bortezomib directly affects BMSCs, and found that IL-6 levels were augmented when only the supernatant obtained from the culture medium of MM.1S cells after bortezomib administration was added to LP101 without MM.1S cells (Fig. 2h). Therefore, we suggest that some kind of material [17] secreted from dead MM cells acted on BMSCs. We analyzed IL-1β as well, because IL-1β secreted from MM cells is known to be a potent inducer of IL-6 production [18]. However, IL-1β elevation was not observed in the supernatant of the medium with MM cells examined between pre-dose and after the administration of bortezomib (data not shown).

Not only fever following bortezomib administration but also lung injury has been reported [7–9]. Based on the present results, we speculate that lung injury after bortezomib administration might also be caused through a similar inflammatory process in the bone marrow milieu, because macrophages and fibroblasts are also structural elements of the alveolar septa. Corticosteroids exert anti-inflammatory effects in part by abrogating the proliferation of macrophages and blocking the production of pro-inflammatory cytokines such as IL-6 and IFN-γ. In fact, previous reports showed that combination therapy with corticosteroids decreased the incidence of complications caused by bortezomib [7–9].

In our results, there was a tendency toward a correlation between BT and objective responses, and objective responses were observed only in patients who demonstrated elevated serum IFN-γ levels (Table 1), although the number of patients examined was too small to draw any definite conclusions concerning the predictive factor of the clinical response after bortezomib administration. In the same way, we were not able to draw any conclusions concerning the relationship between serum IL-6 levels and the clinical response after bortezomib administration. Because elevations in the levels of serum IL-6 following bortezomib administration were transient, we suggest that a transient elevation in the levels of IL-6 secreted from BMSCs has little effect on the growth of MM cells.

In this preliminary study, the number of patients was too small to draw any definite conclusions. Nevertheless, our experimental results regarding the elevated cytokine levels following bortezomib administration were consistent with our clinical observations. We believe that our findings provide a valuable clue for clinicians who treat patients with MM with proteasome inhibitors to understand the contradictory inflammatory syndromes after bortezomib administration.

In conclusion, our study revealed that fever following bortezomib administration was correlated with pro-inflammatory cytokines such as IL-6 or IFN-γ, which were secreted from macrophages or fibroblasts, respectively. Our results clarified the validity of using corticosteroids together with bortezomib in addition to its effectiveness in combination [1, 3]. Because this investigation was a preliminary study involving a small number of patients, the causal protein for pro-inflammatory cytokines secretion from BMSCs and the relationship among systemic inflammatory symptoms following bortezomib administration and other cytokines should be further investigated.

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ORIGINAL ARTICLE

Functional analysis of cytomegalovirus-specific T lymphocytes compared to tetramer assay in patients undergoing hematopoietic stem cell transplantation

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In order to evaluate whether we could predict reactivation of CMV by monitoring the number of CMV-specific cytotoxic T-lymphocytes (CTL), tetramer analysis was performed in 37 patients who underwent hematopoietic stem cell transplantation (HSCT). The results disclosed that the mean number of CMV-specific CTL at day 30 did not differ among patients who developed CMV antigenemia (22/μl) and those who did not (12/μl). Serial tetramer analysis showed that 21% of the patients had >10/μl CMV-specific CTL at the first detection of CMV antigenemia and 67% of the patients had more than 10/μl CMV-specific CTL at the onset of CMV disease. Intracellular staining upon stimulation by CMV lysates and peptide in patients with CMV colitis revealed that both IFN-γ producing CD4+ and CD8+ lymphocytes were suppressed at the onset of CMV colitis (1.6 and 8/μl), which increased with recovery of the disease (19 and 47/μl). These data suggest that it is difficult to predict CMV reactivation solely by the number of CMV-specific CTL. We suggest that additional functional analysis by intracellular cytokine assay may be useful for immunomonitoring against CMV.

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Keywords: CMV; intracellular IFN-γ; CTL; HSCT; HLA-A02

Introduction

Reactivation of CMV is one of the major complications in patients undergoing hematopoietic stem cell transplantation (HSCT) and is significantly related to morbidity and mortality despite the recent development of potent antiviral medications.[1,2] The decision to administer antiviral therapy is currently based on the clinical risk and the detection of viremia by various methods including PCR for CMV-derived DNA or CMV antigenemia assay. However, treatment with antiviral drugs such as ganciclovir and foscarnet increases the risk for secondary graft failure and other infectious complications due to myelotoxicity. To optimize the therapy with minimum drug exposure, it is important to monitor the recovery of CMV-specific immunity accurately. For this purpose, tetramer-based monitoring of CMV-specific cytotoxic T-cells (CTL) has been widely performed in patients with an HLA-A02 or HLA-B07 serotype.[3–11] Some of the results have demonstrated that the reconstitution of CMV-specific CTL as evaluated by quantitative tetramer to levels >10–20/μl is adequate for protection against CMV infection.[5–7] However, some patients with CMV-specific CTL above this level still experience CMV reactivation.[9] It has also been reported that the cellular response to CMV in immunosuppressed patients reflects functional impairment,[10] and CMV reactivation following HSCT has been shown to be associated with the presence of dysfunctional CMV-specific T-cells.[11] Therefore, by itself, the quantification of CMV-specific CTL seems to be insufficient and a simultaneous qualitative analysis of CMV-specific lymphocytes is needed. Furthermore, it is essential that we should develop a universal monitoring method, which is not limited to HLA to cover larger populations, since an epitope that is potent enough for immunomonitoring is not obtained in some HLA types such as HLA-A24.[12] In this study, simultaneous functional analysis of CMV-specific lymphocytes by intracellular cytokine assay upon stimulation with CMV lysate and antigen peptide were performed with tetramer-based CTL quantification in patients who underwent HSCT to identify an optimal monitoring system.

Materials and methods

Study patients

CMV seropositive patients with an HLA-A*0201 or HLA-A*0206 genotype who had undergone allogeneic non-T-cell

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depleted-HSCT between February 2002 and May 2005 were included in this study. Patients were eligible with the availability for 160 days of follow-up. The study was approved by the Ethics Committee and a written informed consent was given by all patients. Peripheral blood samples were obtained at days 30 ± 7 and 60 ± 7 after transplantation. When patients agreed to additional sampling, additional samples were obtained every 2–3 weeks. The median age of studied patients was 52 (21–68). The genotype for HLA-A02 in 37 eligible patients was HLA-A*0201 in 20 patients, HLA-A*0206 in 16 patients and both the HLA-A*0201 and HLA-A*0206 genotypes in one patient. Nine patients received BMT from an unrelated donor, two received BMT from a related donor and the remaining 26 received peripheral blood HSCT from a related donor. With regard to the conditioning regimen, 11 patients received a conventional regimen that included 120 mg/kg CY plus 16 mg/kg BU or 120 mg/kg CY plus 12 Gy of TBI, whereas 26 received a reduced-intensity regimen with 0.66 mg/kg cladribine (2-chlorodeoxyadenosine) plus 8 mg/kg BU or 180 mg/m^2 fludarabine plus 8 mg/kg BU. For patients who received a graft from an unrelated donor or DNA-mismatched donor, 4 Gy of TBI or 5 mg/kg of rabbit antithymocyte globulin (ATG) were added to reduced-intensity conditioning.

Diagnostic tests for CMV infection and CMV disease

CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. All patients and 31 donors (84%) were seropositive for CMV. CMV antigenemia was monitored weekly after engraftment to day 60, and at longer intervals thereafter, by using the immunocytochemical detection of pp65 antigen in leukocytes. Test results were considered to be positive when more than one cell per 50 000 leukocytes was positively stained. CMV disease was diagnosed clinically, with confirmation by biopsy of the involved organ. Pre-emptive antiviral therapy was given with an antigenemia of more than 10 positive cells per 50 000 leukocytes, which we defined as high antigenemia. The initial therapy was ganciclovir 5 mg/kg once per day, which was adjusted according to the follow-up CMV antigenemia value.

Peptide and CMV antigen

A >80% pure HLA-A02-binding peptide NLVPMVATV (AA 495–503, referred to as NLV peptide) from the CMV pp65 phosphoprotein was obtained using high-performance liquid chromatography (Qiagen, Tokyo, Japan).

Tetramer staining

Tetramer staining was performed as recently described.[13] Briefly, 5 µl CD8-FITC, CD4-PC5, CD19-PC5, CD13-PC5 and 2 µl PE-conjugated tetrameric HLA-A*0201 NLV peptide complex (CMV-tetramer), purchased from Beckman Coulter Inc. (Fullerton, CA, USA), were added to 100 µl heparinized blood and incubated for 30 min. After RBC were lysed and washed twice, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). More than 20 000 cells in the lymphocyte gate were acquired and analyzed using Cellquest software. The CD4−, CD19−, CD13− and CD8+ CMV-tetramer-positive fraction of the lymphocyte gate was defined as CMV-specific CTL.

Intracellular cytokine assay

Intracellular cytokine staining was performed as recently described[14] with the following modifications. Peripheral whole blood (1 ml) was stimulated for 6 h at 37 °C with 10 µg/ml NLV peptide or 1 µg/ml CMV lysate (Advanced Biotechnologies, Colombia, MD, USA), in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson, 1 µg/ml each). Breferdin A (Sigma, St Louis, MO, USA; 10 µg/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 µg/ml staphylococcal enterotoxin B or phosphate-buffered saline. Samples were lysed, permeabilized and stained with 2.5 µl CD69-FITC, 20 µl IFN-γ-PE, 0.6 µl CD3-APC and 10 µl CD8− or CD4− PerCP. More than 10 000 cells in the lymphocyte gate were acquired and analyzed using an FACS Calibur. The cells were gated on the CD3+ fraction of the lymphocyte gate and the proportion of IFN-γ and CD8 or CD4 was analyzed. CD69 was used as a marker for activated T-cells.

Statistical analysis

The difference between groups was compared with the Wilcoxon–Mann–Whitney *U*-test and the probabilities of $P<0.05$ were defined as statistically significant.

Results

Tetramer staining

CMV antigenemia was observed in 27 patients (73%) between day 23 and day 56 (median, day 34) after transplantation; 13 (35%) of them had a peak antigenemia level of >10/50 000 leukocytes (high antigenemia) which required ganciclovir therapy and four (11%) subsequently developed CMV disease. The median number of leukocytes and lymphocytes were 3500 (1300–17 200)/µl and 576 (228–3333)/µl at day 30 and 3900 (1400–9700)/µl and 1018 (192–6790)/µl at day 60, respectively. The median percentages of CD4+ and CD8+/lymphocytes were 35% (7–64%) and 38% (20–83%) at day 30 and 25% (6–37%) and 52% (27–83%) at day 60, respectively.

The tetramer analysis showed that the mean and median number of CMV-specific CTL at day 30 was, respectively, 11 and 1.9/µl for patients without CMV antigenemia, 23 and 7.8/µl for those with antigenemia, 33 and 15/µl for those with peak antigenemia <10/50 000, 12 and 3.7/µl for those with high antigenemia, and 21 and 2.4/µl for those who developed CMV disease. There was no significant correlation between the number of CMV-specific CTL and the incidence or severity of CMV antigenemia ($P>0.05$) (Figure 1).

To further evaluate the accurate number of CMV-specific CTL at the onset of CMV antigenemia, serial analysis of CMV-specific CTL was performed weekly in 14 patients (Figures 2 and 3). Patient's characteristics are shown in Table 1. CMV antigenemia was observed in 12 patients, and five of them (UPN1-5) developed high

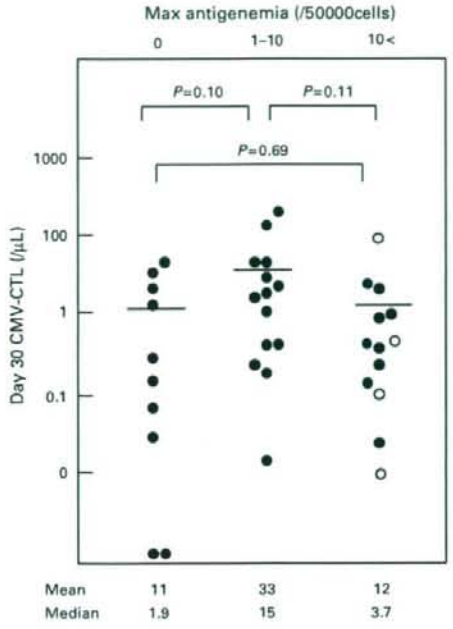

Figure 1 The number of CMV-specific CTL as evaluated by tetramer assay on day 30 post transplantation. The number of CMV-specific CTL did not differ between patients who did not develop CMV antigenemia, who had antigenemia below 10/50 000, who had antigenemia of >10/50 000. The outlined circle ○ indicates patients who developed CMV colitis.

antigenemia, including three (UPN1-3) with CMV colitis. The mean and median number of CMV-specific CTL at the first detection of CMV antigenemia was 21/μl and 4.7 (0–100)/μl in the 12 patients, and three (UPN2, 13, 14) showed >10/μl. For those who did not require antiviral therapy (UPN6-14), the number of CMV-specific CTL was widely ranged. While UPN6-8 showed <10/μl throughout the observation time, the maximum CTL count was >200/μl for UPN12-14. The number of CMV-specific CTL for UPN1 and UPN2 who developed CMV colitis showed >10/μl, which was 14 and 80/μl when diarrhea occurred, and 88 and 63/μl, respectively at the time of colon biopsy which proved CMV colitis.

It has been demonstrated that in patients coexpressing HLA-A02 and HLA-B07, CMV-specific cellular immune responses restricted by HLA-B07 dominate those restricted by HLA-A02, possibly because CD8+ T cells specific for dominant epitopes are able to suppress immune responses to less favored epitopes.[3] The allele frequency of HLA-B07 is low (5.2%) among Japanese[15] and only one patient coexpressed HLA-B07 in this study. We did not exclude this patient (UPN14) from the analysis because the number of HLA-A02-restricted CMV-specific CTL in this patient was 9.5/μl on day 30 and the maximum value reached 243/μl on day 128 suggesting that the coexpression of HLA-B07 seems not to have affected the immunoresponse of HLA-A2 in this patient.

Intracellular cytokine assay

Upon stimulation with CMV lysate, intracellular IFN-γ staining among five patients (UPN1–5) who developed high

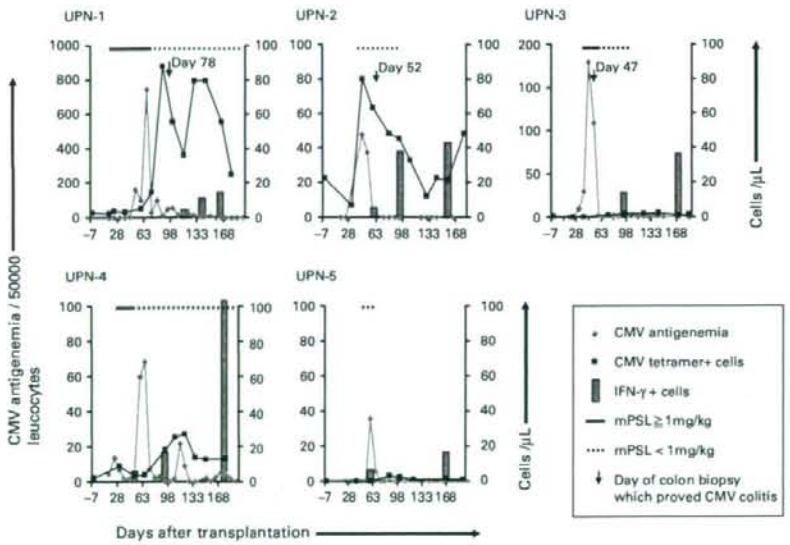

Figure 2 Serial analysis of patients who had high antigenemia of >10/50 000. ■ indicates CMV-specific CTL as evaluated by tetramer assay, ◆ indicates CMV antigenemia, gray bar indicates the number of IFN-γ+cells/μl peripheral blood when stimulated with CMV lysate, the solid line indicates methylprednisolone administration of 1 mg/kg/day or more, the dashed line indicates corticosteroid administration less than 1 mg/kg/day and ↓ indicates the day of colon biopsy which CMV disease was diagnosed. UPN1, 2, 3 developed CMV disease. Intracellular IFN-γ was undetectable on day 60 and day 90 for UPN1 and on day 60 for UPN3.

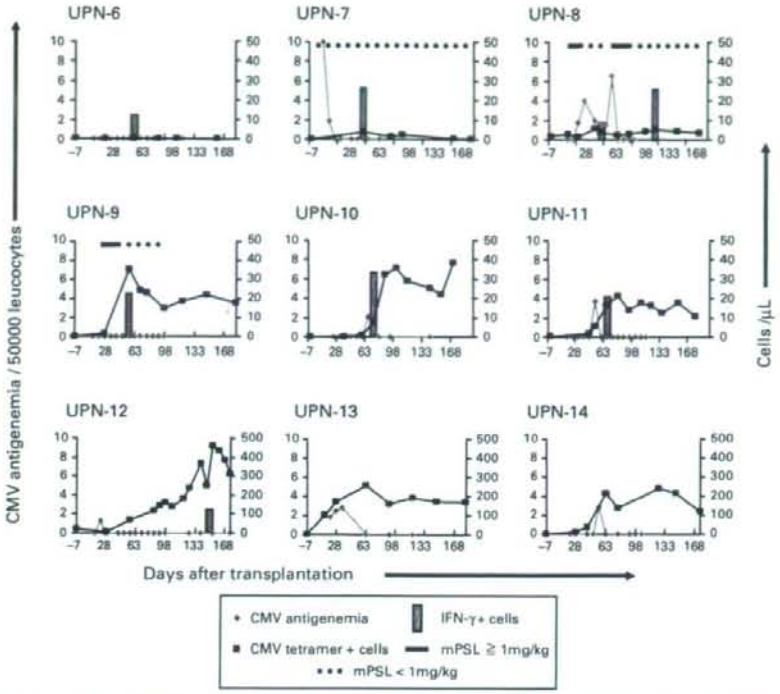

Figure 3 Serial analysis in patients with CMV antigenemia of $<10/50\,000$ or patients without CMV antigenemia. The legends are the same as Figure 2. Intracellular cytokine was not assessed for UPN13 and UPN14.

Table 1 Patients' characteristics

ID	*Age*	*HLA-A locus*	*Primary disease*	*Conditioning regimen*	*GVHD prophylaxis*	*Stem cell source*	*CMV serology*		*Max CMV-Ag*	*CMV disease*
							Recipient	*Donor*		
UPN-01	63	0201, 0206	CML (AP)	CdA/BU	CSP→TAC	PB	+	+	740	+
UPN-02	57	0201	NHL (DLBCL)	CdA/BU	CSP	PB	+	+	48	+
UPN-03	49	0201	NHL (low grade)	CdA/BU	CSP→TAC	PB	+	+	178	+
UPN-04	54	0206	MCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	68	–
UPN-05	59	0206	AML	CdA/BU/TBI	CSP+sMTX	UBM	+	+	35	–
UPN-06	66	0206	MDS (RA)	Flu/BU	CSP+sMTX	PB	+	–	0	–
UPN-07	61	0201	NHL (low grade)	Flu/BU/ATG	CSP	UBM	+	+	10	–
UPN-08	62	0201	AML	CdA/BU	TAC	PB	+	+	6.5	–
UPN-09	43	0201	MDS (RA)	BU/CY	CSP+sMTX	UBM	+	–	0	–
UPN-10	41	0206	AML	BU/CY	CSP+sMTX	RBM	+	+	2.1	–
UPN-11	54	0201	NHL (low grade)	Flu/BU	CSP+sMTX	PB	+	+	3.7	–
UPN-12	32	0206	RCC	CdA/BU	CSP	PB	+	+	2.8	–
UPN-13	42	0206	PCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	2.8	–
UPN-14	43	0206	RCC	CdA/BU/ ATG	CSP	PB	+	+	1.3	–

Abbreviations: ATG = antithymocyte globulin; CdA = cladribine; CML (AP) = CML (accelerated phase); CSP = cyclosporine; DLBCL = diffuse large B-cell lymphoma; Flu = fludarabine; MCL = mantle cell lymphoma; MDS (RA) = myelodysplastic syndrome (refractory anemia); NHL = non-Hodgkin lymphoma; PB = peripheral blood; PCL = plasma cell leukemia; RBM = related bone marrow; RCC = renal cell carcinoma; sMTX = short term methotrexate; TAC = tacrolimus; UBM = unrelated bone marrow.

antigenemia and required antiviral therapy showed that the mean number of IFN-γ-producing cells was 3.6 (0–6.7)/μl at day 60, which subsequently increased to 72 (15–250)/μl at day 160. As for three patients with CMV colitis (UPN1–3), only one patient (UPN2) had detectable level of IFN-γ-producing cells (4.8/μl) at the time of disease

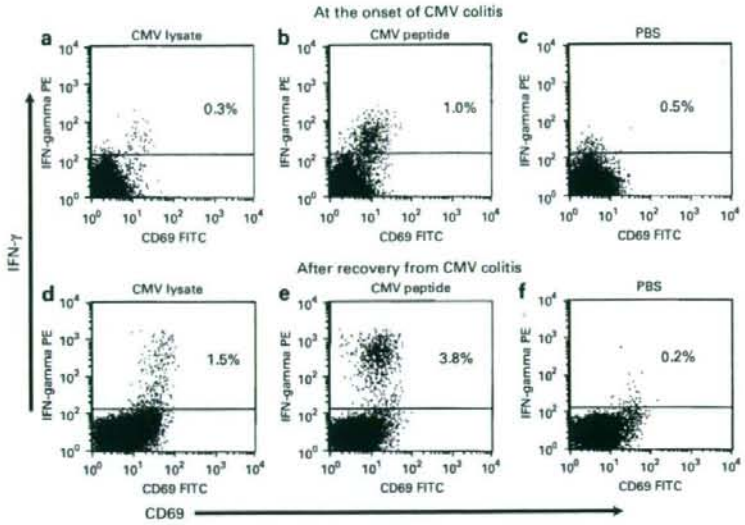

Figure 4 Intracellular cytokine assay in a patient with CMV colitis (UPN2). The samples were taken at the onset of CMV colitis (**a**–**c**) and after recovery from CMV colitis (**d**–**f**). The numbers of IFN-γ-producing cells on lysate stimulation (**a**, **d**) and peptide stimulation (**b**, **e**) both increased after recovery from CMV colitis. (**c**) and (**f**) are negative controls.

onset and were undetectable for the other two patients, which remained negative until day 90 for UPN1. The mean number of IFN-γ+ cells subsequently increased to 19 (5–38)/μl after recovery from CMV disease (Figures 2, 4a and d). Among the patients who did not require antiviral therapy, the IFN-γ-producing cells were all >10/μl at day 60.

When stimulated with CMV peptide, IFN-γ-producing cells numbered 8 (0–16)/μl at the time of disease onset with a subsequent increase to 47 (15–95)/μl after recovery from CMV disease (Figures 4b and e).

Regarding the phenotype of IFN-γ-producing cells, median of 81% (76–100) were CD4+ and <20% were CD8+ upon stimulation by CMV lysate. The staining of IFN-γ was brighter in CD4+ than in CD8+ cells and CD69 was positive for both CD4+ and CD8+ fraction. IFN-γ-producing cells were CD69 low positive and median of 42% (25–68) were CD8+, while the rest were CD8−/CD4− phenotype upon CMV peptide stimulation.

Discussion

Our results showed that it is difficult to predict CMV infection by the number of CMV-specific CTL alone as this did not correlate with the incidence and severity of CMV infection. While UPN1 and UPN2 developed CMV colitis after the recovery of sufficient number of CTL, UPN6, UPN7 and UPN8 did not require antiviral therapy despite low CMV-specific CTL. These results showed that CMV disease could occur after HSCT even in patients with >10/μl CMV-specific CTL as evaluated by tetramer assay, which has been considered to be sufficient to protect against CMV infection.[5,7]

CMV-specific CTL emerged immediately following the detection of antigenemia in most patients, suggesting that CMV infection can be a trigger for the recovery of CMV-specific immunity. However, UPN9 had recovery of CMV-specific CTL at day 60 even though his CMV antigenemia and CMV DNA as evaluated by PCR were negative throughout the course.

On the other hand, intracellular analysis revealed that IFN-γ production in both CD4+ and CD8+ T lymphocytes was depressed in patients with high antigenemia or CMV disease and this had subsequently recovered at disease resolution. Functional analysis methods for CMV-specific immune response by flow cytometry have been established,[16] and it was reported that patients who developed CMV disease after SCT had no detectable IFN-γ production by CD3+/4+ T-cells upon CMV AD-169 antigen stimulation.[17] It has also been demonstrated that levels of IFN-γ-producing CD4+ cells less than one cell/μl and CD8+ less than three cells/μl upon stimulation by CMV-infected autologous dendritic cells are not protective against recurrent infection.[18] As assessed by IFN-γ ELISPOT assay, the threshold level for protection against CMV reactivation was estimated as over one cell/μl peripheral blood upon CMV pp65 peptide stimulation.[19] The number of IFN-γ-producing cells upon CMV lysate stimulation were above ten cells/μl among patients whose antigenemia was <10/50 000 cells in our study, which may be sufficient for protection against CMV reactivation. It is difficult to determine the exact threshold level for protection against CMV since IFN-γ production differs among various stimulating agents. Also the magnitude of response is higher in the cytokine flow cytometry assay while the cytokine flow cytometry assay was less likely than the ELISPOT assay to detect low-level responses.[20]

Several studies on HIV-infected patients have shown the availability of analyzing the phenotype and other cytokine production of virus-specific T-cells such as IL-2, TNF-α.[21,23] It has been demonstrated that virus-specific T-cells, which produce both IFN-γ and IL-2 are important in virus-specific immunity, and that IFN-γ/IL-2 secreting CD8+ T-cells were CD45RA−/CCR7− phenotype and correlated with that of proliferating T-cells, whereas single IFN-γ-secreting cells were either CD45RA−/CCR7− or CD45RA+/CCR7−.[22] Another study has shown that immunorestored patients had increased levels of circulating CMV-specific CD8+ T-cells with 'early' (CD27+/CD28+/CD45RA+, CD27+/CD28+/CD45RA−) and 'intermediate' (CD27−/CD28+/CD45RA−) phenotype.[23] Only IFN-γ production was assessed in our study, however higher-order flow cytometry might have added more discriminatory value. Foster *et al.*[24] demonstrated that CMV-specific CD4+ T-helper cells show the same reconstitution kinetics as CD8+ CTL. Thus, functional analysis of lymphocytes upon lysate stimulation that can be used to assess both CD4+ and CD8+ cells is a useful tool for monitoring T cell immunity against CMV in patients after HSCT. This method is more widely applicable than peptide stimulation or tetramer assay, since it is not restricted to HLA or a single epitope. However, peptide stimulation and tetramer assay may still be a major procedure in the analysis of CD8+ T-cells, since tetramers are widely applied to adoptive immunotherapy of CMV[25] and the dominant population of IFN-γ-producing cells upon lysate stimulation was CD4+. Previous study has demonstrated that flow cytometry following stimulation of PBMC with pp65 and immediate early (IE)-1 peptide pools consisted of 15-aa peptides was highly sensitive and specific in predicting the presence of recognized epitope in the respective proteins.[26] Furthermore, it has been shown that IE-1-specific responses were more important in protective immunity than pp65-specific responses in heart and lung transplant recipients.[27] The stimulation with comprehensive peptide pools might have better assessed both functional CD4+ and CD8+ T-cell responses. Further study is needed to identify whether IE-1 is more important than pp65 in allogeneic HSCT patients, and the significance of IE-1 in Japanese population with low allele frequency of HLA-A1 (1.8%), -B7 (5.2%) or -B8 (<1%),[15] which is known to present IE-1 epitopes.

It is likely that the patients who did not have CMV reactivation despite low CMV-specific CTL had sufficient T-cell immune-recovery against CMV since the number of intracellular IFN-γ positive cells upon CMV lysate stimulation was as high as that in patients who had recovered from CMV reactivation. As for CD8+ T cells in these patients, CTL against other CMV-epitopes besides NLV might have helped to protect against CMV. It is reported that the recovery of CMV specific T-cells is earlier in patients who received reduced-intensity conditioning compared to conventional regimen and this was delayed by the use of ATG.[19,28] Additionally, the graft source and CD3+ T-cell dose significantly influence the recovery of CMV-specific immunity.[28] The difference of immune recovery according to the conditioning regimen and graft source was not demonstrated in this study, probably due to heterogeneous patients and small sample size. Functional depression of the lymphocytes due to corticosteroid for GVHD seems to be the major cause of CMV infection as documented in all patients with high antigenemia. Moreover, 75% of the patients with CMV disease were receiving more than 1 mg/kg/day of methylprednisolone (mPSL), while among those who did not require antiviral therapy, only 13% had received 1 mg/kg/day or more mPSL. The influence of corticosteroid on the number of CMV-specific CTL is controversial. Some studies have reported that a significant reduction of CMV-specific CTL occurred with corticosteroid therapy.[6,8] Others have shown that the frequency and the absolute number of CMV-specific CD8+ T cells were similar in patients receiving corticosteroids and those who didn't, while the CMV-specific CD8+ T cells showed decreased cytokine production.[10,11] Our result was consistent with the latter observation that while the number of CMV-specific CTL does not decrease significantly with corticosteroid therapy, IFN-γ production of CMV-specific CTL is severely suppressed. Therefore, concomitant assessment of T-cell function is essential in patients after HSCT, especially in those who are receiving corticosteroid therapy.

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Infectious complications in chronic graft-versus-host disease: a retrospective study of 145 recipients of allogeneic hematopoietic stem cell transplantation with reduced- and conventional-intensity conditioning regimens

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Abstract: To assess infectious complications associated with chronic graft-versus-host disease (cGVHD) after allogeneic hematopoietic stem cell transplantation (HSCT) with reduced- and conventional-intensity conditioning regimens (RIC, $n = 91$; CIC, $n = 54$, respectively), we retrospectively analyzed data from 145 consecutive patients with cGVHD after allogeneic HSCT from a human leukocyte antigen-matched related or unrelated donor. In the present retrospective analysis, 57% (83/145) of patients with cGVHD developed infections, with a mortality rate of 27% (22/83). The incidences of bacteremia ($n = 28$), central venous catheter-related infections ($n = 11$), bacterial pneumonia ($n = 4$), invasive aspergillosis ($n = 7$), and adenoviral hemorrhagic cystitis ($n = 8$) were significantly higher in patients with prednisolone dose ≥ 1 mg/kg at the time of diagnosis of cGVHD. The present results suggest that infections associated with cGVHD, especially after high-dose prednisolone, are predictive of poor outcome regardless of whether the patient received RIC or CIC.

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Key words: infectious complication; chronic graft-versus-host disease; allogeneic hematopoietic stem cell transplantation; reduced-intensity conditioning; HLA-matched donor

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Infectious complications contribute to morbidity and mortality following allogeneic hematopoietic stem cell transplantation (HSCT). Well-known factors affecting susceptibility to infections include donor type, conditioning regimen, development of graft-versus-host disease (GVHD), and environmental factors. Reduced-intensity conditioning (RIC) regimens are thought to lower the risk of infections because they involve relatively little damage to vital organs (1). However, our experience indicates that with both RIC and conventional-intensity conditioning (CIC) regimens, the incidence of bacterial infections during neutropenia and *Aspergillus* infections is high after allogeneic HSCT (2, 3). Thus, it appears that RIC alone is not sufficient to improve the safety of allogeneic HSCT.

GVHD and the treatment of GVHD with immunosuppressive drugs are also well-known predominant risk factors for the development of opportunistic infections (4–6). In the case of acute GVHD, inpatients can be given comprehensive prophylaxis, including environmental control, to prevent infections over the short term. In contrast, chronic GVHD (cGVHD) is most often a late complication of allogeneic HSCT, and is usually treated on an outpatient basis. Consequently, the resources that can be used to control infections in patients with cGVHD are limited, and prophylaxis should be considered as a long-term approach, taking into account the safety and emergence of drug-resistant pathogens. In Japanese patients, the incidence of cGVHD after allogeneic HSCT is reportedly as high as 50%, with 20% of those who develop cGVHD contracting concurrent infections (7). At present, more transplantation procedures are being performed with peripheral blood stem cell (PBSC) products, in older patients, and with

unrelated donors. The available evidence suggests that all of these factors would result in greater numbers of patients with cGVHD. Thus, management of cGVHD is one of the greatest challenges to physicians practicing HSCT.

In the present study, we evaluated infectious complications associated with cGVHD in patients who received an RIC or a CIC regimen before undergoing PBSC transplantation (PBSCT) from a human leukocyte antigen (HLA)-matched relative (related PBSCT) or bone marrow transplantation (BMT) from an HLA-matched unrelated volunteer (unrelated BMT).

Patients and methods

Patient characteristics

We retrospectively analyzed data from 145 consecutive adult patients with hematologic malignancies who had received allogeneic HSCT with an RIC ($n = 91$) or CIC ($n = 54$) regimen between January 2000 and December 2004 at our institution. All of these 145 patients had sustained engraftment, had survived for >100 days following transplantation, and had developed cGVHD. The following types of patients were excluded: patients who suffered from disease progression before the development of cGVHD and received donor lymphocyte infusion, and patients with a history of previous allogeneic HSCT. Significant differences were observed between the RIC and CIC groups in terms of the age of the patients and donors, the gender of the patients, diagnosis, disease risk (8), time from diagnosis to transplantation, donor type and source of stem cells, and GVHD prophylaxis. The patient characteristics are summarized in Table 1. Typing for HLA-A, -B, and -DR antigens of the donor and recipient was performed using low-resolution DNA typing. The frequency with which allogeneic PBSCT is performed in Japan has been increasing since it became eligible for reimbursement from health insurance organizations in the year 2000, and our banking system only approves donation of bone marrow. The clinical characteristics of cGVHD, including use of immunosuppressive drugs at diagnosis and initial treatment, are summarized in Table 2. The present study was approved by the Ethics Committee of our institution, and all 145 subjects provided informed consent.

Conditioning regimen and supportive care

The CIC regimen consisted of cyclophosphamide (CY, 120 mg/kg), in combination with either 12 Gy total-body irradiation (TBI, $n = 25$) or busulfan (BU, 16 mg/kg; $n = 29$). The RIC regimen consisted of BU (8 mg/kg) in combination with either fludarabine (Flu, 180 mg/m^2; $n = 70$) or 2-chlorodeoxyadenosine (2-CdA, 0.66 mg/kg; $n = 21$); 14 patients received either anti-thymocyte globulin (ATG, 5–10 mg/kg; $n = 6$) or 4 Gy TBI ($n = 8$). All patients received cyclosporine (CSP, 3 mg/kg/day; $n = 137$) or tacrolimus (TAC, 0.03 mg/kg/day; $n = 8$), with ($n = 78$) or without ($n = 67$) short courses of methotrexate (MTX; related PBSCT, 10 mg/m^2 on day 1, and 7 mg/m^2 on days 3 and 6; unrelated BMT, 10 mg/m^2 on days 3, 6, and 11) as GVHD prophylaxis. All patients received prophylactic ciprofloxacin (200 mg orally 3 times daily) for prevention of infections until neutrophil recovery. Trimethoprim-sulfamethoxazole (80 mg of trimethoprim once daily) was administered for the prevention of *Pneumocystis* pneumonia and encapsulated bacterial infection, from the first day of the conditioning regimen until day 3, and from day +30 until 6 months after transplantation, or for prolonged periods in patients with cGVHD. Patients also received oral or intravenous fluconazole (100 mg once daily) for prevention of infection by *Candida* species, and low-dose acyclovir (600 mg until engraftment, and then 100 mg/day orally), starting at the same time as the conditioning regimens and continuing until cessation of administration of immunosuppressive drugs (9). Cytomegalovirus (CMV) antigenemia was monitored weekly until cessation of the administration of immunosuppressive drugs. Testing for CMV antigenemia consisted of direct immunoperoxidase staining of leukocytes with a peroxidase-labeled monoclonal antibody. Quantitative real-time polymerase chain reaction was not performed.

Definition of outcome

Patients with grades II–IV acute GVHD were treated with prednisolone (PSL) according to a standard regimen (10). Chronic GVHD was assessed and graded according to the standard criteria (11). The diagnosis and staging of cGVHD were also assessed according to the working report published by the National Institutes of Health Consensus Development Project (12). Relapse was defined either by morphologic evidence of the disease in the peripheral blood, marrow, or extramedullary sites, or by recurrence and persistence of pre-transplant chromosomal abnormalities in cytogenetic analysis of the marrow cells.

Infectious complications

A documented infection was defined as signs and symptoms associated with microbiological documentation of a pathogen from the site of infection. Culture-documented bacteremia, fungemia, or viremia was considered to be a definite infection, regardless of symptoms. On the other hand, clinical infection was defined as signs or symptoms consistent with an infection, but without microbiological confirmation. Central venous catheter (CVC)-related