

Figure 3. Correlation between the expansion of V α 24⁺ V β 11⁺ NKT cells and the expression of CD1d on CD14⁺ cells. We analyzed the association between the fold expansion of V α 24⁺ V β 11⁺ NKT cells and CD1d expression on CD14⁺ cells in 16 samples (10 HD and six ML patients) after G-CSF administration. There was a strong correlation between these two factors both in HD and ML patients ($r^2 = 0.84$).

patient CD14⁺ cells with CD14⁺ cells from a HD, which have an increased intensity of CD1d presentation, might restore the expansion of the ML patient's V α 24⁺ NKT cells. However, as shown in Figure 6, replacement of a patient's CD14⁺ cells with an alternative donor's CD14⁺ cells did not exert effective proliferation recovery of V α 24⁺ NKT cells (Figure 6). On the other hand, the replacement of a donor's CD14⁺ cells with a patient's CD14⁺ cells caused an increased expansion compared with the reversed condition, but it was not statistically significant (Figure 6). The intensity of CD1d expression on CD14⁺ cells used in this experiment was 25.88% for HD and 2.79% in ML patients.

Discussion

We have previously reported that G-CSF treatment can enhance the expansion of V α 24⁺ NKT cells in volunteer donors [18,19]. Based on these results, we are attempting to develop a protocol for the adaptive transfer of cultured NKT cells from the PB MNC of a G-CSF-mobilized HD to a patient. The incidence of V α 24⁺ NKT cells in cancer patients is suppressed compared with the healthy state [12,16,21]. Our results showed that the expansion capability of V α 24⁺ NKT cells isolated from the MNC of G-CSF-mobilized ML patients could not be restored, although the reason remains unclear. The frequency of V α 24⁺ NKT cells in ML patients was not significantly suppressed, but the expansion of V α 24⁺ NKT cells was severely disturbed in ML patients compared with HD. These results suggest that the number of V α 24⁺ NKT cells in a patient's PB is almost the same as that of a normal donor, but the patient's V α 24⁺ NKT cells have lost the potential to expand in response to α GalCer stimulation after G-CSF stimulation. Based on our results, one of the causes of this difference appears to be the intensity of CD1d expression. The expression of CD1d molecules on CD14⁺ monocytes was augmented after G-CSF mobilization in HD but not in ML patients. This intensity of CD1d might be affected by G-CSF and other inhibitory factors in cancer-bearing host cells such as transforming growth factor (TGF)- β [22]. The V α 24⁺ NKT cell expansion efficiency strongly correlated with the intensity of CD1d expression ($r^2 = 0.82$). Xu *et al.* [23] suggested the expression of CD1d was essential for the development of NKT cells, and Smyth *et al.* [4] reported that in tumor immunity

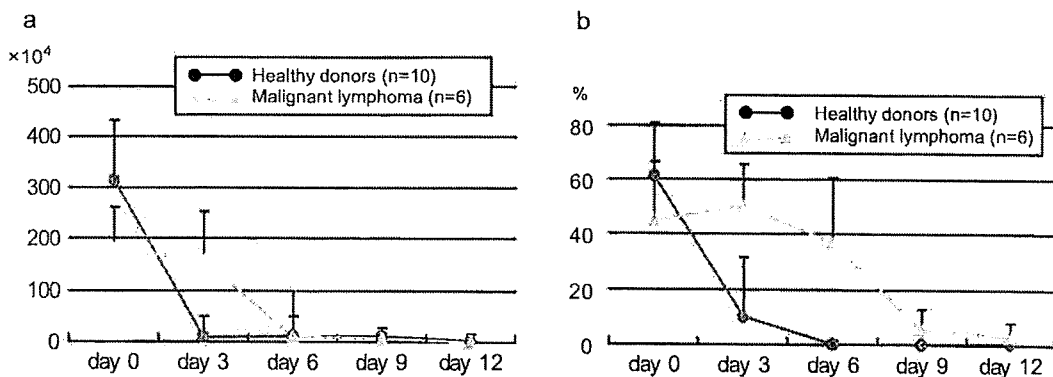


Figure 4. Prolonged survival of patients' CD14⁺ cells in the culture process. We assessed the cell culture subpopulation kinetics of cells from 10 HD (CD1d expression 25.25 \pm 10.50%) and six ML patients (CD1d expression 1.40 \pm 1.56%). The CD14⁺ cells survived longer in ML patients than in HD both in (a) absolute number and (b) proportion of CD14⁺ cells. The error bars indicate the standard deviation.

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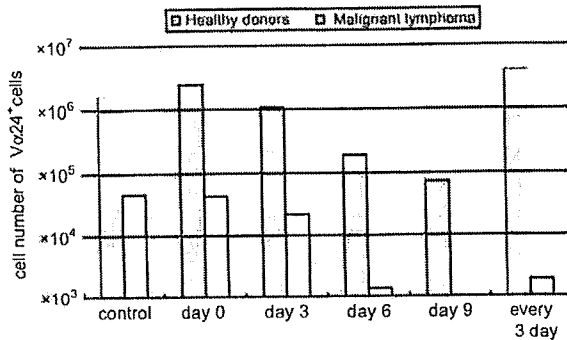


Figure 5. Repeated stimulation of CD14⁺ cells led to reversed results in accordance with the intensity of CD1d expression. CD14⁺ cells were added to the culture system every 3 days or on day 0, 3, 6 or 9. The repeated addition of CD14⁺ cells (CD1d expression 25.00%) promoted the expansion of Vα24⁺ Vβ11⁺ NKT cells from healthy subjects. However, the repeated initiation of CD14⁺ cells (CD1d expression 1.96%) from ML patients suppressed the expansion of Vα24⁺ Vβ11⁺ NKT cells. This result is representative of four confirmed cases.

the recognition of self-ligand CD1d in the presence of IL-12 was crucial for the activation of NKT cells. These reports suggest that CD1d plays various non-redundant roles in the selection, differentiation, expansion and activation of NKT cells.

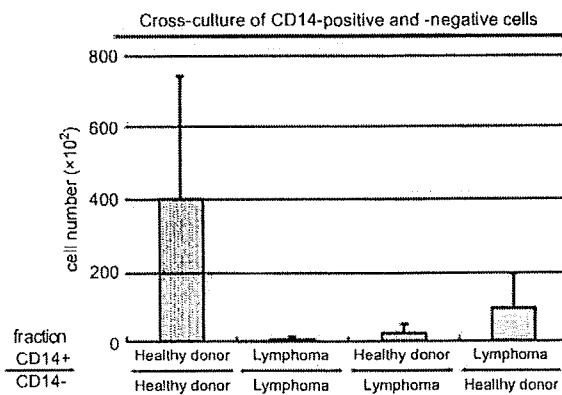


Figure 6. CD14⁺ cells derived from HD did not augment the expansion of Vα24⁺ NKT cells from an ML patient. Replacement of a patient's CD14⁺ cells with a HD's CD14⁺ cells (CD1d expression 25.88%) did not promote effective proliferation recovery of Vα24⁺ NKT cells. Conversely, replacement of a donor's CD14⁺ cells with a patient's CD14⁺ cells (CD1d expression 2.79%) caused an increased expansion, but it was not statistically significant (n = 4; four independent experiments were performed).

Because CD14⁺ cells are essential for the expansion of Vα24⁺ NKT cells [18], while CD20⁺ cells are not [20], we supposed that CD1d expression on CD14⁺ cells, not CD20⁺ cells, contributes to Vα24⁺ NKT cell expansion. We also confirmed that the depletion of B cells did not influence the expansion of Vα24⁺ NKT cells (data not shown). Interestingly, the monocytes from ML patients survived in the culture medium longer than monocytes from HD during *ex vivo* culture and, unexpectedly, those monocytes suppressed the expansion of Vα24⁺ NKT cells from HD. The patients' monocytes showed low expression levels of the CD1d molecule. This reduced expression level might be one reason for the long survival of patient monocytes in the culture medium, resulting in the low expansion efficacy of Vα24⁺ NKT cells *in vitro*. Our results showed longer survival of patient monocytes and suggested that the surviving monocytes disturbed Vα24⁺ NKT cell expansion. We have not yet identified the mechanisms by which patients' monocytes disturb the Vα24⁺ NKT cell expansion.

Focusing on the Vα24⁺ NKT cell by itself, it was suggested that the suppression of Vα24⁺ NKT cell expansion in ML patients after G-CSF treatment is the result of the diminished potentiality of Vα24⁺ NKT cells, because CD14⁺ cells derived from HD did not retain the efficacy of Vα24⁺ NKT cell expansion in ML patients. It has been reported that the lost potentiality of Vα24⁺ NKT cells might vary among different malignancies [21], and research to evaluate the CD1d expression and Vα24⁺ NKT cell expansion in other types of cancer is important. In the present study, we used MNC collected from ML patients. In our preliminary experiments, MNC obtained from colon cancer and renal cell carcinoma patients showed the expansion of Vα24⁺ NKT cells, although the expansion capacities were lower than those of healthy volunteers. Crough *et al.* [21] reported the same observation, and these results suggest that the degree of deterioration in Vα24⁺ NKT cell expansion is different in various types of cancers. This observation suggests that Vα24⁺ NKT cell expansion is poor in patients with hematologic malignancies, such as ML. We also know that the expression of CD1d molecules on CD14⁺ cells from solid tumors is suppressed, as is the expansion efficacy of Vα24⁺ NKT cells (data not shown). These results indicate that the suppressed expression of CD1d is a significant reason for the insufficient expansion of NKT cells in patients with hematologic tumors or solid tumors. The

immunologic background of solid tumor patients was genetically normal. If the diminished expansion of V α 24⁺ NKT cells in solid tumors is the result of CD1d inhibition on CD14⁺ cells, CD14⁺ cells obtained from HD could provide complete recovery of the V α 24⁺ NKT cell expansion in solid cancer patients; the addition of 'good' CD14⁺ cells could be a simple way to overcome the poor expansion of V α 24⁺ NKT cells from cancer patients.

There are several reports suggesting that the suppressive factors in V α 24⁺ NKT cell expansion in cancer patients are multiple and complicated. Other inhibitory factors, such as various cytokines [24–27], cellular factors [28] and further signaling pathways [29], should be revealed for efficient expansion. Although our data are insufficient to develop an effective V α 24⁺ NKT cell expansion system for adoptive immunotherapy in cancer patients, our findings are informative for allograft-based NKT cell therapy.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan.

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ORIGINAL ARTICLE

Allo-SCT using reduced-intensity conditioning against advanced pancreatic cancer: a Japanese survey

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Pancreatic cancer is a frequent cause of cancer-related mortality and has an extremely poor prognosis. To evaluate the efficacy of allogeneic hematopoietic SCT with reduced-intensity conditioning (RICT) against pancreatic cancer, we analyzed the clinical data of 22 patients. After a fludarabine-based conditioning regimen followed by the infusion of PBSCs, all but two achieved engraftment. Complete, partial and minor response was observed in 1, 2 and 2 patients, respectively, with an overall response rate of 23%. Median survival was only 139 days and the major cause of death was tumor progression. Poor performance status before RICT and a lower number of infused CD34-positive cells were associated with shorter survival after RICT. Patients who developed chronic GVHD tended to survive longer than those who did not. These findings support the investigation of a novel treatment strategy to enhance the immunological effect against pancreatic cancer.

Bone Marrow Transplantation (2008) 42, 99–103;
doi:10.1038/bmt.2008.94; published online 7 April 2008
Keywords: reduced-intensity conditioning; SCT; mini-transplantation; pancreatic cancer; graft-versus-tumor effect

Introduction

Allogeneic hematopoietic SCT is an established treatment for a variety of hematological disorders. However, its application has been limited to young patients because of various complications including regimen-related toxicities, GVHD, infection and so on. Therefore, SCT with reduced-intensity conditioning (RICT) has been investigated for use

in older or clinically infirm patients. The antitumor effect of this therapeutic approach depends not only on the antineoplastic agents and/or irradiation in the conditioning regimen, but also on the immunological graft-versus-tumor effect after RICT.¹ Although RICT has not been clearly shown to have a clinical advantage over conventional chemotherapy, some studies have suggested that RICT may be beneficial in elderly patients with hematological malignancies.²

Since the late 1990s, several studies of RICT against advanced solid tumors have been performed to harness the graft-versus-tumor effect.³ A clinical tumor response after RICT was observed in several solid tumors, especially in renal cell cancer and breast cancer.^{4–6} Pancreatic cancer is the fifth most common cause of cancer-related mortality in Japan and the United States, and carries an extremely poor prognosis. The median duration of survival in advanced pancreatic cancer is less than 6 months, even when patients are treated with gemcitabine.⁷ The combination of gemcitabine with the other chemotherapeutic agents failed to significantly improve survival.^{8–10} Furthermore, although the combination of gemcitabine and erlotinib, a molecular targeting agent against epidermal growth factor receptor, significantly prolonged survival, the difference in median survival was only 2 weeks.¹¹ Because of this poor prognosis by chemotherapy, treatment strategies to enhance immunological effects against pancreatic cancer have been investigated. One of these is a vaccination targeting tumor-specific antigens such as CA19-9 and CEA.¹² Another strategy is RICT to harness a strong allogeneic immunological antitumor effect. The first successful application of RICT against pancreatic cancer was reported in 2001.¹³ Several other reports have suggested the existence of an immunological graft-versus-tumor effect against pancreatic cancer, but the number of patients in each report was too small to draw any meaningful conclusion.^{14,15} Therefore, we collected the clinical results of RICT against pancreatic cancer from transplantation centers in Japan, in which a prospective clinical trial of RICT against pancreatic cancer had been performed.

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Received 28 January 2008; revised 28 February 2008; accepted 5 March 2008; published online 7 April 2008

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/m²/day for 6 days), BU (4 mg/kg/day for 2 days) and gemcitabine (1000 mg/m²/day for 3 days) at the University of Tokyo Hospital, whereas the combination of fludarabine (30 mg/m²/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/m²/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/mm³ 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

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

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

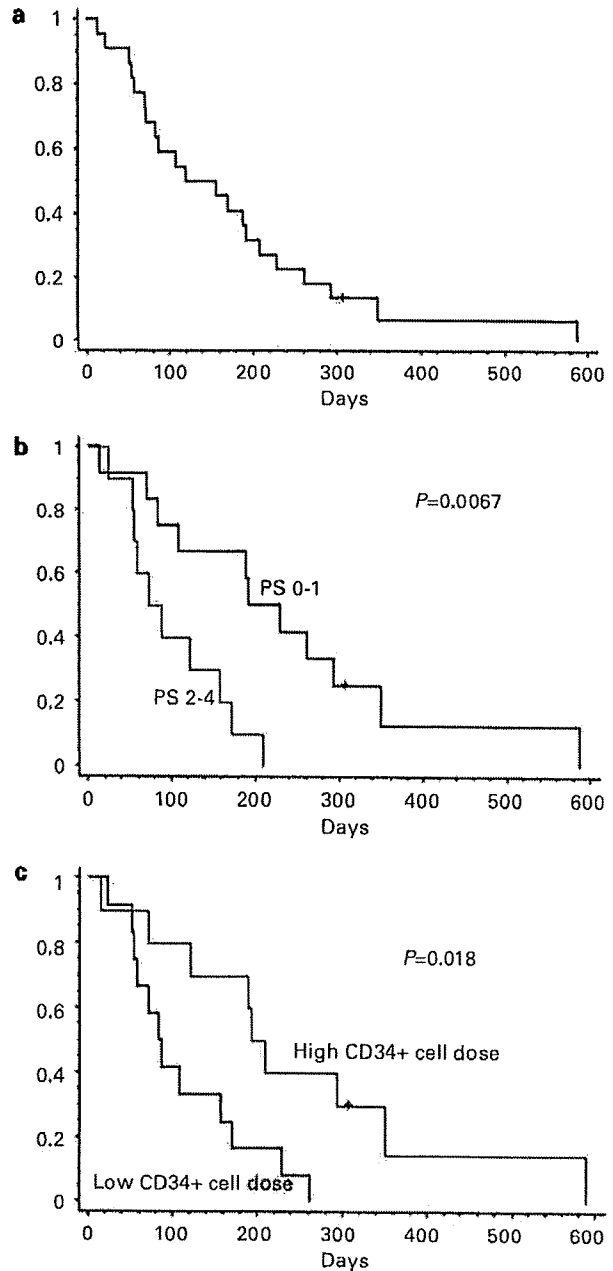


Figure 1 Patient survival, overall (a) and grouped according to risk factors (b and c).

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

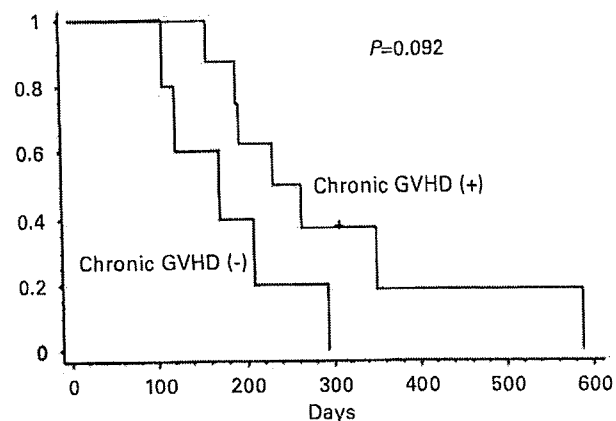


Figure 2 Overall survival of patients who survived at least 100 days after transplantation grouped according to the presence or absence of chronic GVHD.

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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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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Received: 28 December 2007 / Revised: 13 August 2008 / Accepted: 4 September 2008 / Published online: 7 November 2008
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Abstract Bortezomib blocks the activation of nuclear factor- κ B-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.

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-prednisone 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)- κ B-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

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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/m² ($n = 2$), 1.0 mg/m² ($n = 2$), and 1.3 mg/m² ($n = 6$) 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 $\geq 37.5^{\circ}\text{C}$, 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/m² cohort, both in the 1.0 mg/m², and five of six in the 1.3 mg/m². 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 (D-S)	Stage 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/m² and one at 1.3 mg/m²) 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

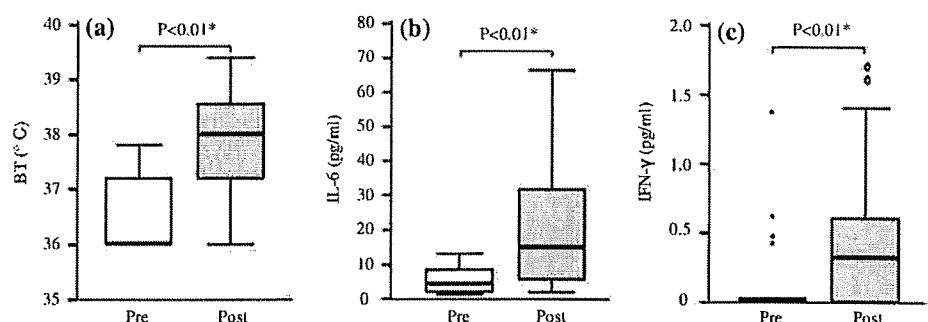
alone (Fig. 2a), bortezomib increased IL-6 levels in the supernatant of LP101 cells, which originated from macrophages, when co-cultured with MM cells except for U266 (Fig. 2b) or without MM cells (Fig. 2c). Similarly, a marked elevation of IFN- γ levels was induced by adding bortezomib to AA101, which 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- κ B-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



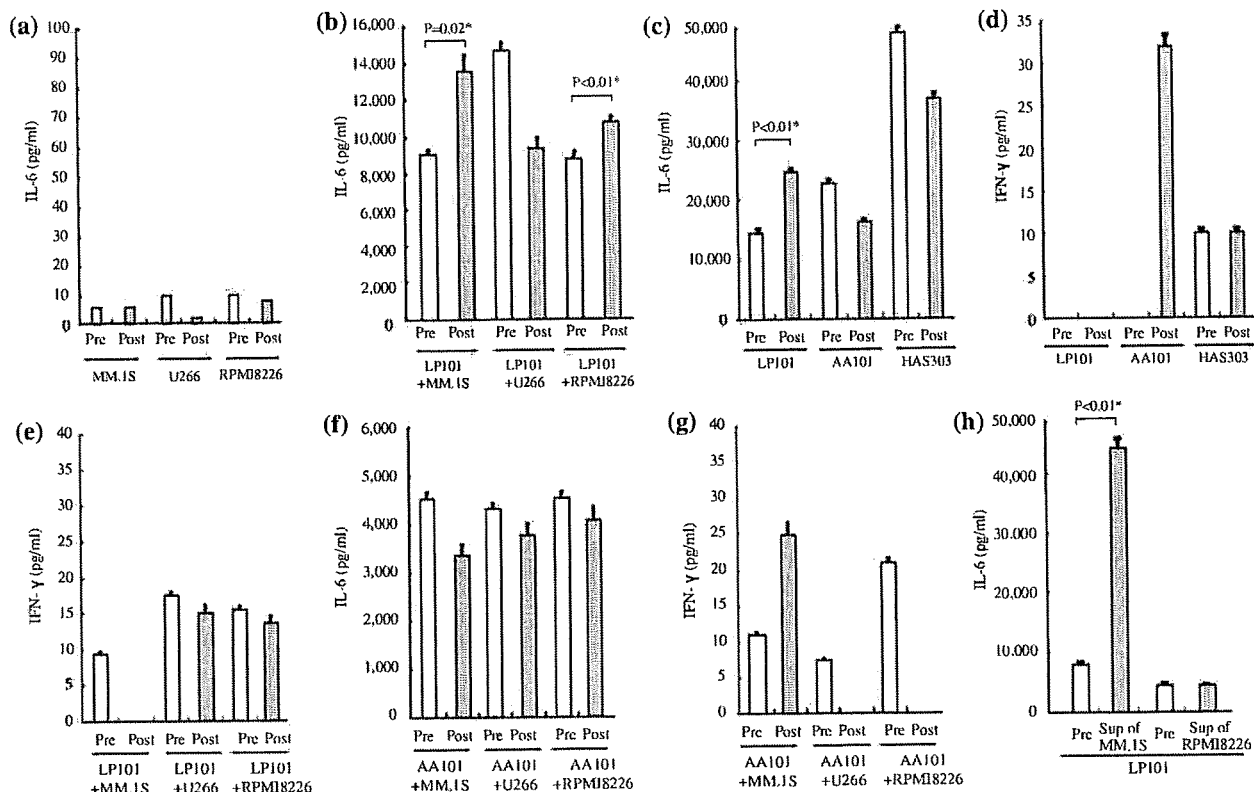


Fig. 2 Levels of cytokines in various combinations between multiple myeloma (MM) and bone marrow stromal cells. The elevation of IL-6 levels in the supernatant was not observed after the addition of bortezomib to any examined MM cells alone (a). Bortezomib augmented IL-6 secretion from LP101 cells when 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 \pm 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 StepOnePlusTM 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-1 β as well, because IL-1 β secreted from MM cells is known to be a potent inducer of IL-6 production [18]. However, IL-1 β 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.

Acknowledgments We thank Janssen Pharmaceutical K.K. for supporting the measurement of serum cytokines of the patients enrolled in this study. We also thank Ms. Junko Nomoto, a medical technologist of the Hematology Division at the National Cancer Center Hospital, for her helpful advice regarding PCR methods. This work was supported in part by a Grant-in-Aid for Cancer Research (17-17) from the Ministry of Health, Labor and Welfare, Tokyo, Japan (Takashi Watanabe).

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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/ μ l) and those who did not (12/ μ l). Serial tetramer analysis showed that 21% of the patients had >10/ μ l CMV-specific CTL at the first detection of CMV antigenemia and 67% of the patients had more than 10/ μ l 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/ μ l), which increased with recovery of the disease (19 and 47/ μ l). 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.

Bone Marrow Transplantation (2008) 41, 515–521; doi:10.1038/sj.bmt.1705932; published online 19 November 2007
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/ μ l 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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Received 7 August 2007; revised 10 October 2007; accepted 15 October 2007; published online 19 November 2007

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/m² 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 μ l CD8-FITC, CD4-PC5, CD19-PC5, CD13-PC5 and 2 μ l PE-conjugated tetrameric HLA-A*0201 NLV peptide complex (CMV-tetramer), purchased from Beckman Coulter Inc. (Fullerton, CA, USA), were added to 100 μ l 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 described¹⁴ with the following modifications. Peripheral whole blood (1 ml) was stimulated for 6 h at 37 °C with 10 μ g/ml NLV peptide or 1 μ g/ml CMV lysate (Advanced Biotechnologies, Colombia, MD, USA), in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson, 1 μ g/ml each). Breferrdin A (Sigma, St Louis, MO, USA; 10 μ g/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μ g/ml staphylococcal enterotoxin B or phosphate-buffered saline. Samples were lysed, permeabilized and stained with 2.5 μ l CD69-FITC, 20 μ l IFN- γ -PE, 0.6 μ l CD3-APC and 10 μ l 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)/ μ l and 576 (228–3333)/ μ l at day 30 and 3900 (1400–9700)/ μ l and 1018 (192–6790)/ μ l 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/ μ l for patients without CMV antigenemia, 23 and 7.8/ μ l for those with antigenemia, 33 and 15/ μ l for those with peak antigenemia <10/50 000, 12 and 3.7/ μ l for those with high antigenemia, and 21 and 2.4/ μ l 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

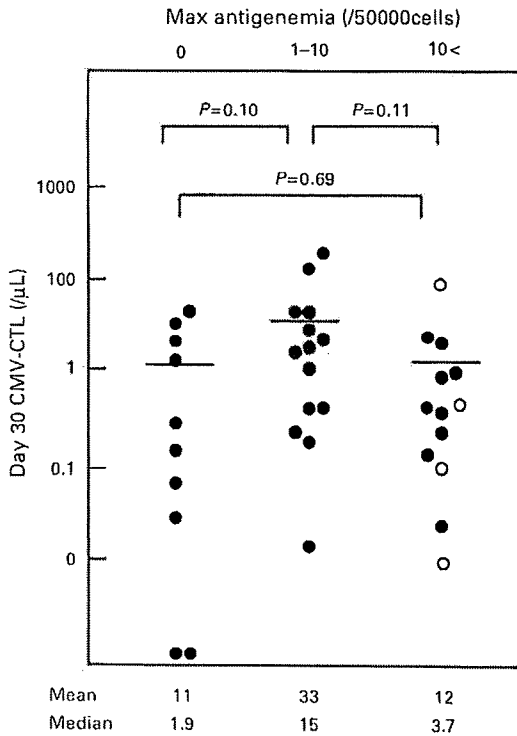


Figure 1 The number of CMV-specific CTL as evaluated by tetramer assay on day 30 post transplantation. The number of CMV-specific CTL did not differ between patients who did not develop CMV antigenemia, who had antigenemia below 10/50 000, who had antigenemia of > 10/50 000. The outlined circle ○ indicates 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/ μ L and 4.7 (0-100)/ μ L in the 12 patients, and three (UPN2, 13, 14) showed >10/ μ L. For those who did not require antiviral therapy (UPN6-14), the number of CMV-specific CTL was widely ranged. While UPN6-8 showed <10/ μ L throughout the observation time, the maximum CTL count was >200/ μ L for UPN12-14. The number of CMV-specific CTL for UPN1 and UPN2 who developed CMV colitis showed >10/ μ L, which was 14 and 80/ μ L when diarrhea occurred, and 88 and 63/ μ L, 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 Japanese¹⁵ 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/ μ L on day 30 and the maximum value reached 243/ μ L 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

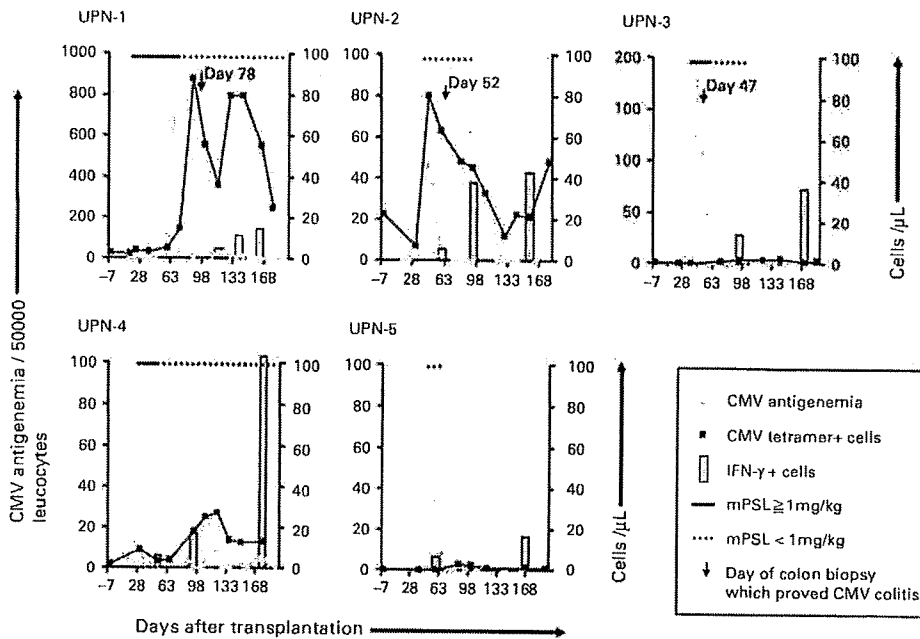


Figure 2 Serial analysis of patients who had high antigenemia of > 10/50 000. ■ indicates CMV-specific CTL as evaluated by tetramer assay, ◆ indicates CMV antigenemia, gray bar indicates the number of IFN- γ + cells/ μ L peripheral blood when stimulated with CMV lysate, the solid line indicates methylprednisolone 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.

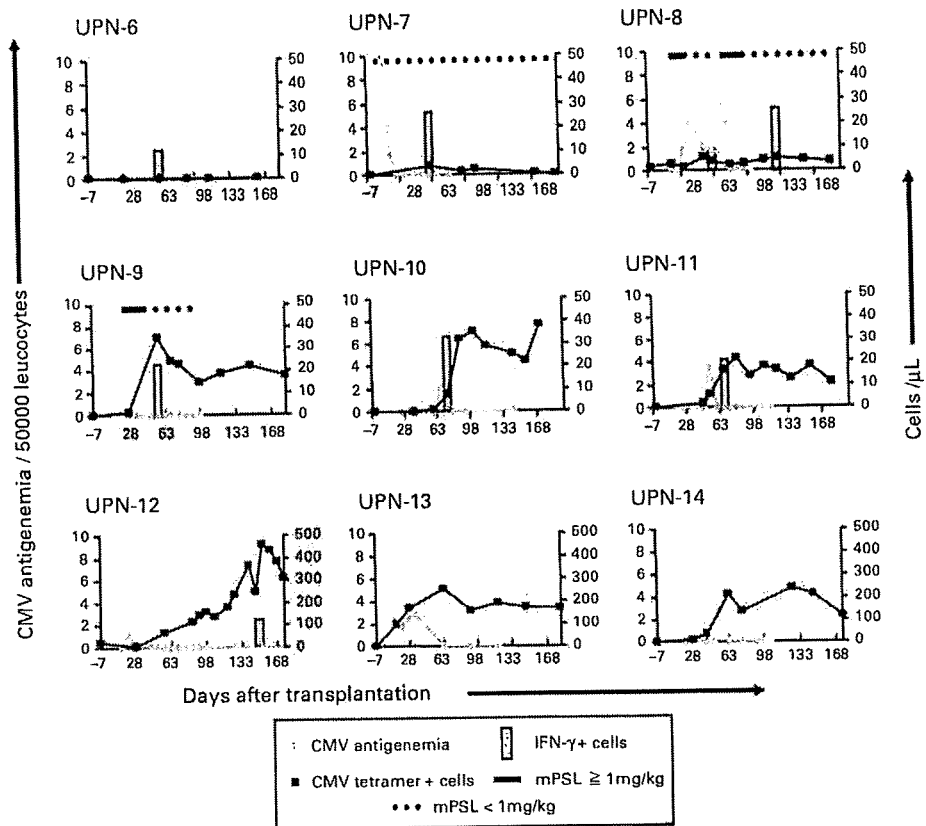


Figure 3 Serial analysis in patients with CMV antigenemia of <10/50000 or patients without CMV antigenemia. The legends are the same as Figure 2. Intracellular cytokine was not assessed for UPN13 and UPN14.

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)/ μ l at day 60, which subsequently increased to 72 (15-250)/ μ l

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/ μ l) at the time of disease