**ORIGINAL ARTICLE**

# Reduced-intensity unrelated donor bone marrow transplantation for hematologic malignancies

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**Abstract** To review a current experience of unrelated bone marrow transplantation (BMT) with reduced-intensity conditioning (RIC) regimens, we conducted a nationwide survey with 77 patients (age, 25–68 years). The backbone RIC regimen was a combination of fludarabine or cladribine, busulfan or melphalan and total body irradiation at 2–4 Gy. Five patients died early, but 71 (92%) achieved initial neutrophil recovery. Thereafter, 36 patients (47%) died of therapy-related complications, 23 (30%) of whom died within day 100. Grades II–IV acute graft-versus-host disease (GVHD) occurred in 34 of the 68 evaluable patients (50%). In a multivariate analysis, a regimen containing antithymocyte globulin (ATG) was significantly associated with a decreased risk of acute GVHD ($P = 0.041$). Thirty-three patients are currently alive with a median follow-up of 439 days (28–2002 days), with an OS of 50% at 1 year. In conclusion, unrelated BMT with RIC regimens can be a curative treatment in a subset of patients.

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# 1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a possible curative approach for patients with various hematologic malignancies. Recently, the application of reduced-intensity conditioning (RIC) regimens, mostly incorporating fludarabine as a backbone agent, has been explored for patients whose age or concomitant medical conditions contraindicate the use of conventional myeloablative regimens [1–3]. Since only 30–40% of patients have an appropriate family donor available [4], the establishment of an unrelated donor transplantation program with RIC regimens is urgently needed.

Graft rejection, regimen-related toxicities and graft-versus-host disease (GVHD) have been the major problems in unrelated HSCT with RIC [5–13]. In unrelated transplantation, engraftment is influenced by the source of stem cells and superior results have been observed with peripheral blood stem cells (PBSC) compared to bone marrow [9, 14]. Nevertheless, PBSC has not yet been approved as a graft source for unrelated transplantation in Japan [15]. The level of regimen-related toxicities directly depends on the intensity of the regimen, and the incidence of GVHD increases with unrelated donors compared to related donors. Although attempts have been made to overcome these problems, a suitable procedure for unrelated bone marrow transplantation (BMT) with RIC regimens has not yet been established. To accumulate further expertise, we conducted a nationwide survey of Japanese patients with hematologic malignancy who had undergone BMT from an HLA-matched or -mismatched unrelated donor with RIC regimens. Although the present data were obtained from a limited population of patients, these findings may show a current status of unrelated BMT with RIC.

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# 2 Patients and methods

## 2.1 Data sources

This survey collected the data of 77 consecutive patients in 17 participating hospitals who received unrelated BMT with RIC for hematologic malignancies between 2000 and 2004. Data were derived from questionnaires distributed to each hospital. Additional questionnaires were sent to confirm the follow-up data, including the occurrence of GVHD. The minimum data required for inclusion of a patient in this study were age, sex, histological diagnosis, status at transplant, donor information, conditioning regimen, date of transplant, donor chimerism status, therapy-related complications, date of last follow-up, disease status at follow-up, date of disease progression (PD)/death and cause of death.

This study was approved by institutional review board of each individual center. All patients provided written informed consent according to the Declaration of Helsinki. Unrelated donors provided consent through the Japan Marrow Donor Program as part of its standard procedures. The indications, conditioning regimens, management of GVHD and supportive care for BMT were left to the discretion of each institution. Patients who had previously received allogeneic HSCT and those younger than 20 years were not included. Patients younger than 50 years who had organ dysfunction and/or have previously received high-dose chemotherapy with autologous HSCT were also included.

## 2.2 Definitions

RIC regimens were defined as previously reported [6, 9, 10], and conditioning regimens that included either beyond 4 Gy of total body irradiation (TBI), 8 mg/kg of busulfan or 140 mg/m$^2$ of melphalan were excluded from the study. Alleles at the HLA-A, -B, and -DRB1 loci were identified by middle-resolution DNA typing as described previously [16]. Risk status at transplantation was categorized as either standard risk or high risk. Standard-risk diseases included acute leukemia in first complete remission, chronic myeloid leukemia in first chronic phase, and refractory anemia of myelodysplastic syndrome (MDS). Other diseases were categorized as high-risk disease. Graft failure was analyzed in patients who survived more than 28 days posttransplant according to the criteria reported by Petersdorf et al. [17]. Briefly, the definition included failure of the absolute neutrophil count (ANC) to surpass 500/mm$^3$ before relapse, death or second transplantation, as well as a decrease in the ANC to less than 100/mm$^3$ on at least three consecutive determinations with a finding of severe hypoplastic marrow. The degree of donor chimerism among peripheral blood T cells was assessed several times

between day 28 and day 100 after HSCT using fluorescence in situ hybridization (FISH) to detect X and Y chromosomes for recipients of grafts from sex-mismatched donors, and polymerase chain reaction-based analyses of polymorphic microsatellite regions for recipients of sex-matched or sex-mismatched transplants. Mixed chimerism was defined as the detection of 5–90% of donor cells in the peripheral blood. Acute and chronic GVHD were graded according to the consensus criteria [18, 19]. Patients who survived 100 days were evaluable for the assessment of chronic GVHD. Overall survival (OS) was measured as the time from the day of transplantation until death from any cause, and progression-free survival (PFS) was the time from the day of transplantation until PD/relapse or death from any cause. Patients who died from transplantation-related causes were classified as non-relapse mortality (NRM) regardless of their disease status.

## 2.3 Statistical analysis

The primary endpoint of this study was OS and chimerism. The secondary endpoints were PFS, NRM, PD, and the incidence of acute and chronic GVHD. Descriptive statistical analysis was performed to assess patient baseline information. Patients were divided into two groups: age 60 or above and less than 60. OS and PFS were calculated using the Kaplan–Meier method. The cumulative incidence of acute GVHD was calculated using the method described by Gooley et al. [20] to eliminate the effect of competing risks. The competing event for acute GVHD was defined as death without grades II–IV acute GVHD. For each endpoint, a Cox proportional hazard model was used for univariate and multivariate analyses. The factors included in the analysis were HLA disparity (mismatch vs. identical), recipient age (age 60 or above vs. less than 60), use of TBI (yes vs. no), use of ATG (yes vs. no), diagnosis of AML (yes vs. no), risk status (high vs. standard) and acute GVHD (II–IV vs. 0–I). Acute GVHD in the model was treated as a time-varying covariate. We defined statistical significance as a $P$ value less than 0.05. All statistical analyses were performed using STATA version 8 (College Station, TX).

## 3 Results

### 3.1 Patients and diagnoses

The patients' characteristics are listed in Table 1. The median age of the patients was 54 years (range, 25–68 years) as a whole. Twenty-one patients (27%) had acute myelogenous leukemia (AML), 2 (3%) had acute lymphoblastic leukemia, 5 (7%) had chronic myeloid leukemia, 20 (26%) had MDS or myeloproliferative disease (refractory anemia,

$n = 8$; refractory anemia with excess blasts, $n = 9$; others, $n = 3$), 19 (25%) had non-Hodgkin lymphoma (follicular lymphoma, $n = 12$; diffuse large B-cell lymphoma, $n = 4$; mantle cell lymphoma, $n = 2$; peripheral T-cell lymphoma, unspecified, $n = 1$), 7 (9%) had adult T-cell leukemia/lymphoma, and 3 (4%) had multiple myeloma. Sixty-three patients (82%) had high-risk disease at the time of allogeneic BMT.

### 3.2 Conditioning regimens

Conditioning regimens are shown in Table 2. None received ex vivo T-cell depleted transplantation.

### 3.3 HSCT procedure and supportive care

Forty-seven patients (61%) were transplanted from a matched, 24 (31%) were from a 1 allele-mismatched, and 6 (8%) were from a 2 or 3 allele-mismatched unrelated donor. All patients received bone marrow as a source of stem cells. The prophylaxis of GVHD was either cyclosporine- or tacrolimus-based. Thirty-nine patients (51%) received cyclosporine with methotrexate, including five patients who received an ATG-containing preparative regimen. Nine patients (12%) received cyclosporine alone, including five patients who received ATG. Each patient received cyclosporine with mycophenolate mofetil and cyclosporine with prednisolone, respectively. Twenty-five patients (33%) received tacrolimus with methotrexate, including one patient who received ATG. Two patients (3%) received tacrolimus alone, including one who received ATG. Granulocyte colony-stimulating factor was administered intravenously from day +1 or +6 until neutrophil engraftment in all patients.

### 3.4 Engraftment and chimerism

Five patients died before the engraftment evaluation, with a median survival time of 15 days (range, 2–17 days). Seventy-one patients (92%) achieved initial neutrophil recovery, but three patients (two AMLs and one MDS) later experienced secondary graft failure; one each with AML and MDS after unrelated BMT from an HLA-1 allele-mismatched donor received a second transplantation when they failed to achieve subsequent complete donor-type chimerism, but both died of infectious complications. The other patient with AML after unrelated BMT from an HLA-6 allele-matched donor achieved initial complete chimerism, but later developed secondary graft failure upon the administration of ganciclovir for cytomegalovirus antigenemia. However, this patient achieved the spontaneous recovery of autologous marrow function and is currently surviving beyond 2,000 days.

**Table 1** Patient characteristics

| Variable | Younger than 60 years ($n = 60$) | 60 years or older ($n = 17$) |
|---|---|---|
| Patient age (range, median) | 25–59, 52 | 60–68, 63 |
| Disease | | |
| Acute myelogenous leukemia | 16 (27%) | 5 (29%) |
| Acute lymphoblastic leukemia | 2 (3%) | 0 |
| Chronic myeloid leukemia | 5 (8%) | 0 |
| Myelodysplastic syndrome or myeloproliferative disease | 12 (20%) | 8 (47%) |
| Malignant lymphoma | 16 (27%) | 3 (18%) |
| Adult T-cell leukemia/lymphoma | 7 (12%) | 0 |
| Multiple myeloma | 2 (3%) | 1 (6%) |
| Risk status | | |
| Standard | 13 (22%) | 1 (6%) |
| High | 47 (78%) | 16 (94%) |
| HLA disparity | | |
| Matched | 37 (62%) | 10 (59%) |
| One-mismatched | 19 (32%) | 5 (29%) |
| Two or more mismatched | 4 (7%) | 2 (12%) |
| Donor–recipient sex match | | |
| Male–male | 20 (33%) | 11 (65%) |
| Male–female | 16 (27%) | 2 (12%) |
| Female–male | 9 (15%) | 4 (24%) |
| Female–female | 15 (25%) | 0 |
| GVHD prophylaxis | | |
| Cyclosporine ± methotrexate | 38 (63%) | 10 (59%) |
| Tacrolimus ± methotrexate | 21 (35%) | 6 (35%) |
| Others | 1 (2%) | 1 (6%) |
| Median nucleated cell dose infused ($\times 10^8$/kg, range) | 2.80 (0.39–5.52)[a] | 2.92 (0.76–4.30) |

*HLA* Human leukocyte antigen, *GVHD* graft-versus-host disease

[a] The data of two patients were excluded because infused nucleated cell dose was unknown

Chimerism was evaluated in 68 patients (88%), with short tandem repeats analysis ($n = 52$), variable number of tandem repeats analysis ($n = 5$) and FISH analysis in the case of sex mismatch ($n = 11$). Complete donor chimerism was confirmed in 58 (85%) within day 100. Mixed chimerism was confirmed in nine patients (13%), but two later reverted to recipient type. One patient failed to achieve donor-type chimerism due to disease relapse on day 20. The incidence of complete donor chimerism was similar in those younger and older than 60 years (85 and 86%), with a similar incidence of mixed chimerism (15 and 14%). No patients received donor lymphocyte infusion.

### 3.5 GVHD

Acute GVHD occurred in 41 of the 68 evaluable patients (60%), grades II–IV in 34 (50%) and grades III–IV in 14 patients (21%). Chronic GVHD occurred in 26 of the 42 evaluable patients (62%), with extensive type in 23 (55%). The incidence of grades II–IV acute GVHD was the same in patients younger and older than 60 years (50%). The incidence of grades III–IV acute GVHD (22 and 14%) and extensive chronic GVHD (56 and 50%) was similar. In unrelated BMT, from HLA-6 allele-matched ($n = 40$), HLA-1 allele-mismatched ($n = 23$), and HLA-2 or 3 allele-mismatched ($n = 5$) donors, grades II–IV acute GVHD occurred, respectively, in 18 (45%), 10 (43%) and 3 patients (60%), and chronic GVHD occurred in 15 (38%), 9 (39%) and 2 patients (40%). In univariate and multivariate analyses, an ATG-containing regimen was significantly associated with a decreased risk of the onset of grades II–IV acute GVHD (data not shown).

### 3.6 Survival

Thirty-three patients are currently alive with a median follow-up of 439 days (28–2,002 days), with an OS of 50% at 1 year and 46% at 2 years. The OS of patients younger than 60 years was 49% at 2 years (95% confidence interval [CI], 34–62%), and this could not be defined in older patients (95% CI, 15–45%). Patients younger than 60 years

**Table 2** Conditioning regimens

| Conditioning regimens | Younger than 60 years ($n = 60$) | 60 years or older ($n = 17$) |
|---|---|---|
| TBI-containing | | |
| Fludarabine 180 mg/m$^2$ (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, TBI 4 Gy | 30 (50%) | 6 (35%) |
| Fludarabine 125–180 mg/m$^2$, melphalan 80–140 mg/m$^2$, TBI 4 Gy | 5 (8%) | 3 (18%) |
| Fludarabine 180 mg/m$^2$ (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, TBI 2 Gy | 2 (3%) | 0 (0%) |
| Fludarabine 180 mg/m$^2$, TBI 4 Gy | 0 (0%) | 1 (6%) |
| ATG-containing | | |
| Fludarabine 180 mg/m$^2$ (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, ATG | 5 (8%) | 4 (24%) |
| Fludarabine 180 mg/m$^2$, cyclophosphamide 60 mg/kg, ATG | 1 (2%) | 0 (0%) |
| Fludarabine 180 mg/m$^2$, ATG | 1 (2%) | 0 (0%) |
| TBI and ATG-containing | | |
| Fludarabine 180 mg/m$^2$, oral busulfan 8 mg/kg, TBI 4 Gy, ATG | 1 (2%) | 1 (6%) |
| Non-TBI and non-ATG | | |
| Fludarabine 180 mg/m$^2$, oral busulfan 8 mg/kg | 6 (10%) | 2 (12%) |
| Fludarabine 125–180 mg/m$^2$, melphalan 140 mg/m$^2$ | 5 (8%) | 0 (0%) |
| Fludarabine 180 mg/m$^2$, oral busulfan 8 mg/kg, cyclophosphamide 60 mg/kg | 2 (3%) | 0 (0%) |
| Fludarabine 180 mg/m$^2$, oral busulfan 8 mg/kg, thiotepa 10 mg/kg | 1 (2%) | 0 (0%) |
| Fludarabine 180 mg/m$^2$, cyclophosphamide 60 mg/kg | 1 (2%) | 0 (0%) |

*TBI* Total body irradiation, *ATG* antithymocyte globulin (ATG-Fresenius 10 mg/kg or thymoglobulin 5 mg/kg)

tended to show better survival than older patients ($P = 0.124$). The HLA disparity (match vs. mismatch), TBI vs. non-TBI, ATG vs. non-ATG-containing regimen, and disease category (AML vs. MDS or myeloproliferative disease vs. lymphoid malignancies) was not significantly associated with OS (data not shown). Patients with standard risk tended to show better survival than those with high risk ($P = 0.129$). In univariate and multivariate analyses, no variables were significantly associated with OS (data not shown).

### 3.7 NRM and PD

Thirty-six patients (47%) died of therapy-related complications, with a cumulative incidence of NRM at 1 year of 43% (95% CI, 31–56%). Of the patients who died of therapy-related complications, 23 (30%) died within day 100 of transplantation and 13 (17%) died thereafter. The NRM at 1 year in patients younger and older than 60 years was 38% (95% CI, 25–53%) and 61% (95% CI, 36–85%), respectively, as shown in Fig. 1. The causes of NRM were infection (23%), regimen-related toxicity (14%) and GVHD (9%). GVHD-related mortality was found in 26%. Infection was the major cause of death in patients younger than 60 years. Regimen-related toxicity, mainly pulmonary complications, was the major cause of treatment failure for patients older than 60 years. In univariate and multivariate analyses, no variables were significantly associated with
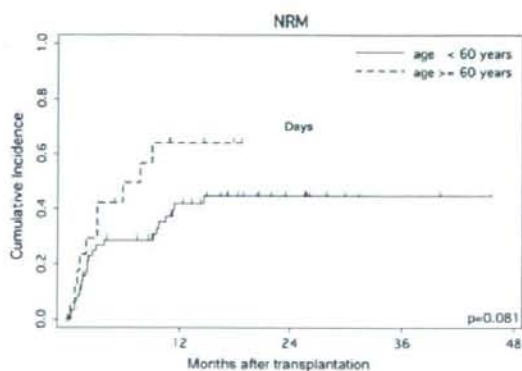


**Fig. 1** Non-relapse mortality stratified according to patient age, younger or older than 60 years

NRM (data not shown). Relapse or progression of primary disease after unrelated BMT with RIC regimens was observed in 13 patients (17%; 10 patients younger than 60 years and 3 older than 60 years). There were no relapsed patients after transplantation in standard risk group. The incidence of death due to relapse or progression of primary disease was 14%. In univariate and multivariate analyses, no variables were significantly associated with PD although patients with grades II–IV acute GVHD showed a relatively lower incidence of PD (data not shown).

## 4 Discussion

This report reviews the current experience of unrelated BMT with RIC regimens in Japan, with particular focus on the risk factors for engraftment, GVHD, NRM, survival and PD. Although the engraftment rate has been reported to be lower when RIC unrelated transplantation was performed with bone marrow compared to peripheral blood cells [9, 10], we observed that sustained engraftment was achieved in 99% of evaluable patients, with complete donor chimerism confirmed in 85%. The incidence of graft failure was not different from that in RIC transplantation from related donors in Japan; 3.7% in recipients with an HLA-matched donor and 5.7% in those with a 1-locus-mismatched donor [21]. Complete donor chimerism in our study was comparable with that reported from the National Marrow Donor Program (85 vs. 84%) [22]. In our study, two-thirds of patients successfully received 2–4 Gy TBI-containing regimens, which were aimed at the enhancement of engraftment, as suggested in a previous report with patients with aplastic anemia [23], while 2 of the 12 patients who received an ATG-containing regimen had late graft failure, similar to a previous report which noted an incidence of 19% [5]. It has been reported that the Japanese population is more homogenous than others in terms of the distribution of HLA. Thus, it would be possible that the impact of minor HLA disparities on engraftment may become prominent after RIC transplantation.

Despite the observed satisfactory engraftment rate, we confirmed a high NRM rate (47%) after unrelated BMT with variable RIC regimens, due mostly to GVHD-related complications, including infections under steroid therapy, as previously designated by Wong et al. [10]. On the other hand, the incidence of death due to relapse or progression of primary disease was low (14%). Hence, successful prophylaxis and treatment of GVHD is particularly important in this procedure, and studies with ATG [5, 24] or alemtuzumab [25–27] have reported encouraging results. Although the number of patients was still small, in our study an ATG-containing regimen resulted in a decreased incidence of acute and chronic GVHD, despite the use of a lower dose (ATG-Fresenius 10 mg/kg or Thymoglobulin 5 mg/kg) than reported elsewhere. This study showed that age older than 60 years tended to be associated with a higher risk of NRM after unrelated HSCT with RIC regimens, though this relation was not statistically significant in a multivariate analysis. This finding, however, is limited by the small sample size. Additional use of ATG may reduce the incidence of GVHD-related NRM even in older patients but ATG should be carefully incorporated since about 20% of patients who received an ATG-containing regimen developed late graft failure in our study.

This study suggested that the onset of grades II–IV acute GVHD was associated with a lower incidence of PD, although this was not statistically significant in a multivariate analysis, possibly due to the small sample size. However, GVHD in turn resulted in a higher incidence of NRM, and a desirable graft-versus-leukemia or lymphoma effect would be offset, particularly in older patients [10, 28]. Hence, our observation echoes the warning that the intentional induction of GVHD should be avoided.

Compared to the long-term follow-up data after unrelated HSCT with RIC from the NMDP reported by Giralt et al. [22], our NRM at 1 year was worse (43 vs. 30%), but OS was likely to be better (50% at 1 year and 46% at 2 years vs. 44% at 1 year, 28% at 3 years and 23% at 5 years). In their report, disease stage, performance status, stem cell source, HLA matching, and timing of transplant were the most important prognostic factors for survival after RIC unrelated donor transplantation. This study suggested that high risk and HLA-mismatched patients were associated with worse OS, although this was not statistically significant in the multivariate analysis. Interpretation of these results, however, should be careful because of relatively short period of follow-up and the small sample size in our study. Although high risk patients was 82%, rate of relapse were unexpectedly low in our study. This might be due to earlier mortality, which precludes estimate of relapse rate. Alternately, more patients (60%) received more intense conditioning composed of 8 mg/kg of busulfan or 80–140 mg/m$^2$ of melphalan and 4 Gy TBI in our study.

In conclusion, we confirmed that unrelated BMT with RIC regimens can be a curative therapeutic option in a subset of patients with advanced hematologic malignancy, but at the expense of a high risk of severe complications and NRM. The incorporation of low-dose TBI may be advantageous for enhancing engraftment, and a suitable prophylaxis for GVHD still remains a primary target of clinical research. Based on the observed data, a prospective trial is currently underway to determine the value of a lower dose of ATG (ATG-Fresenius 5 mg/kg) to be added to the combination of fludarabine and busulfan.

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# Preengraftment Serum C-Reactive Protein (CRP) Value May Predict Acute Graft-versus-Host Disease and Nonrelapse Mortality after Allogeneic Hematopoietic Stem Cell Transplantation

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## ABSTRACT

In a mouse model, inflammatory cytokines play a primary role in the development of acute graft-versus-host disease (aGVHD). Here, we retrospectively evaluated whether the preengraftment C-reactive protein (CRP) value, which is used as a surrogate marker of inflammation, could predict posttransplant complications including GVHD. Two hundred twenty-four adult patients (median age, 47 years; range: 18-68 years) underwent conventional stem cell transplantation (CST, n = 105) or reduced-intensity stem cell transplantation (RIST, n = 119). Patients were categorized according to the maximum CRP value during neutropenia: the "low-CRP" group (CRP < 15 mg/dL, n = 157) and the "high-CRP" group (CRP ≥ 15 mg/dL, n = 67). The incidence of documented infections during neutropenia was higher in the high-CRP group (34% versus 17%, $P = .004$). When patients with proven infections were excluded, the CRP value was significantly lower after RIST than after CST ($P = .017$) or after related than after unrelated transplantation ($P < .001$). A multivariate analysis showed that male sex, unrelated donor, and HLA-mismatched donor were associated with high CRP values. The high-CRP group developed significantly more grade II-IV aGVHD ($P = .01$) and nonrelapse mortality (NRM) ($P < .001$), but less relapse ($P = .02$). The present findings suggest that the CRP value may reflect the net degree of tissue damage because of the conditioning regimen, infection, and allogeneic immune reactions, all of which lead to subsequent aGVHD and NRM.

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## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is associated with high treatment-related mortality (TRM) because of acute graft-versus-host disease (aGVHD) and infections [1,2]. Inflammatory cytokines, for example, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin-1 (IL-1), and IL-6 [3-11], are produced following conditioning and play a primary role in activating T cells, leading to GVHD and resultant target tissue destruction [12,13]. An acute-phase protein, C-reactive protein (CRP), is produced by hepatocytes downstream of IL-6 [14] and is widely used as a reliable

surrogate marker of infectious diseases [15-19]. This process is further stimulated by other cytokines including TNF-$\alpha$ [12,13]. After allogeneic HSCT, the elevation of CRP was observed with infectious complications, but not in uncomplicated aGVHD [8,20]. On the other hand, elevation of CRP has been shown to be associated with TRM [21-24]. Nevertheless, these previous studies adopted the sporadic measurement of CRP and mostly focused on patients undergoing conventional HSCT (CST) with a myeloablative regimen. It has been hypothesized that recently developed reduced-intensity HSCT (RIST) decreases regimen-related toxicities and, hence, may reduce inflammation

that augments the subsequent allogeneic immune reaction to induce GVHD and nonrelapse mortality (NRM).

In this study, the correlation between the preengraftment CRP value and subsequent clinical events was analyzed to test whether high CRP reflected the degree of tissue damage because of the conditioning regimen, infections, and allogeneic immune reactions and/or inflammation, all of which could contribute to subsequent aGVHD and NRM.

## MATERIALS AND METHODS

### Patient Characteristics

The data from a cohort of 224 consecutive adult patients with hematologic malignancies, who were treated between January 2002 and July 2006 at the National Cancer Center Hospital (NCCH, Tokyo, Japan), were reviewed retrospectively. Patients who developed graft failure or who had previous allogeneic transplantation were excluded. Their characteristics are listed in Table 1. The median age of the patients was 47 years (range: 18-68 years), and their diagnosis included acute myeloid leukemia (AML, n = 94), acute lymphoblastic leukemia (ALL, n = 23), non-Hodgkin lymphoma (NHL, n = 62), myelodysplastic syndrome (MDS, n = 27) and chronic myeloid leukemia (CML, n = 12). Standard risk included acute leukemia in first complete remission, chronic leukemia in the first chronic phase, MDS in refractory anemia, and NHL in complete remission, with the rest of the patients categorized as a high-risk group. Stem cell sources used for transplantation included bone marrow (BM, n = 108), peripheral blood stem cells (PBSC, n = 98) and cord blood cells (CB, n = 18). One-hundred five patients received a CST regimen including total-body irridiation (TBI)-based (n = 50) and non-TBI-based busulfan-containing regimens (n = 55), whereas 119 patients received a RIST regimen including fludarabine or cladribine plus busulfan or melphalan (Table 1). CMV serostatus was positive in 157 patients and negative in 67 patients. The median age of the patients was 49 years in the high-CRP group (range: 19-67) and 47 years in the low-CRP group (range: 18- 68). Written informed consent was obtained according to the Declaration of Helsinki.

### Transplantation Procedures

GVHD prophylaxis included cyclosporine- (n = 174) and tacrolimus-based regimens (n = 50), with an additional short course of methotrexate (MTX) in 165 patients. Granulocyte colony-stimulating factor (G-CSF) was administered in all patients from day +6 of transplantation until engraftment was confirmed. Most patients received ciprofloxacin (200 mg orally 3 times daily) for bacterial prophylaxis until neutrophil engraftment. Fluconazole (100 mg once daily)

Table 1. *Patients' Characteristics*

| Variable | N (%)/ Median | | P Value |
|---|---|---|---|
| | Low CRP Group CRP < 15 mg/dL n = 157 | High CRP Group CRP ≥ 15 mg/dL n = 67 | |
| Age (year) | 47 (18-68) | 49 (19-67) | .85 |
| <40 | 53 (34) | 26 (39) | |
| ≥40 | 104 (66) | 41 (61) | .47 |
| Patient sex | | | |
| Male | 84 (54) | 48 (72) | |
| Female | 73 (46) | 19 (28) | .01 |
| Donor sex | | | |
| Male | 81 (52) | 30 (45) | |
| Female | 76 (48) | 37 (55) | .35 |
| CMV serostatus | | | |
| Positive | 140 (89) | 64 (96) | |
| Negative | 17 (11) | 3 (4) | .20 |
| Disease risk | | | |
| Standard | 35 (22) | 17 (25) | |
| High | 122 (78) | 50 (75) | .62 |
| Conditioning | | | |
| CST | 72 (47) | 33 (50) | |
| RIST | 85 (53) | 34 (50) | .64 |
| GVHD prophylaxis | | | |
| Cyclosporin-based | 122 (78) | 52 (78) | |
| Tacrolimus-based | 35 (22) | 15 (22) | .99 |
| Short term MTX (+) | 107 (68) | 58 (87) | .004 |
| Relation to donor | | | |
| Related | 94 (60) | 13 (19) | |
| Unrelated | 63 (40) | 54 (81) | <.001 |
| Stem cell source | | | |
| Bone marrow | 63 (40) | 45 (67) | |
| PBSC | 87 (55) | 11 (16) | |
| Cord blood | 7 (5) | 11 (16) | <.001 |

CRP indicates C-reactive protein; CMV, cytomegalovirus; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; GVHD, graft-versus-host disease; MTX, methotrexate; PBSC, peripheral blood stem cells; HLA, human leukocyte antigen.

was administered for fungal prophylaxis. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and varicella zoster virus until the cessation of immunosuppressive agents. Prophylaxis against *Pneumocystis jiroveci* infection was provided with trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day −3 of transplantation, and from day +28 until day +180 or the discontinuation of immunosuppressive agents. Patients with fever during the neutropenic period were treated with cefepime, and additional agents including vancomycin and aminoglycosides, and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the absolute neutrophil count exceeded $0.5 \times 10^9$/L. In our institute, the CRP level was serially measured as part of our routine checkup at least 3 times a week. Hence, all serially admitted patients were subjected to this analysis. Every patient had started CRP measurement

**Table 2.** *Comparison of Preengrafment CRP Value Stratified According to the Conditioning Regimen (CST versus RIST) and the Relation to Donor (Related versus Unrelated)*

| Patients' Characteristics | CRP Value Median (Range) |
|---|---|
| All patients | 8.9 (0.1-42.7) |
| CST | 10.5 (0.3-31.3)* |
| Related | 9.4 (0.6-30.0)† |
| Unrelated | 10.6 (0.3-31.3)† |
| RIST | 6.2 (0.1-42.7)* |
| Related | 1.6 (0.1-9.7)‡ |
| Unrelated | 16.2 (0.5-42.7)‡ |

CST indicates conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation.
*P = .017.
†P = .33.
‡P < .001.

before the initiation of the conditioning regimen, and the median pretransplant CRP level was 0.3 mg/dL (range: 0.0-20.5 mg/dL). The median maximum CRP value during neutropenia was 8.9 mg/dL (0.1-42.7, Table 2).

The "maximum CRP level" was determined by measuring both the CRP level and the neutrophil count, as shown in the example in Figure 1A. The average number of levels assessed for each patient was 8 (range: 1-30). The median day of the maximum CRP level was day 10 of HSCT (range: 0-25), with 79% of patients developing this in later days ($\geq$8 days). The patients were categorized according to the maximum CRP level after the threshold CRP level was determined following a preliminary analysis of the maximum CRP level after CST using an ROC curve analysis (data not shown). The "low-CRP" group (CRP <15 mg/dL) included 157 patients and the "high-CRP" group (CRP $\geq$15 mg/dL) included 67 patients.

**Statistical Analyses**

The primary endpoint of this study was the occurrence of grade II-IV and grade III-IV aGVHD, according to the Consensus Criteria [25]. The secondary endpoints were overall survival (OS) and nonrelapse mortality (NRM). Standard descriptive
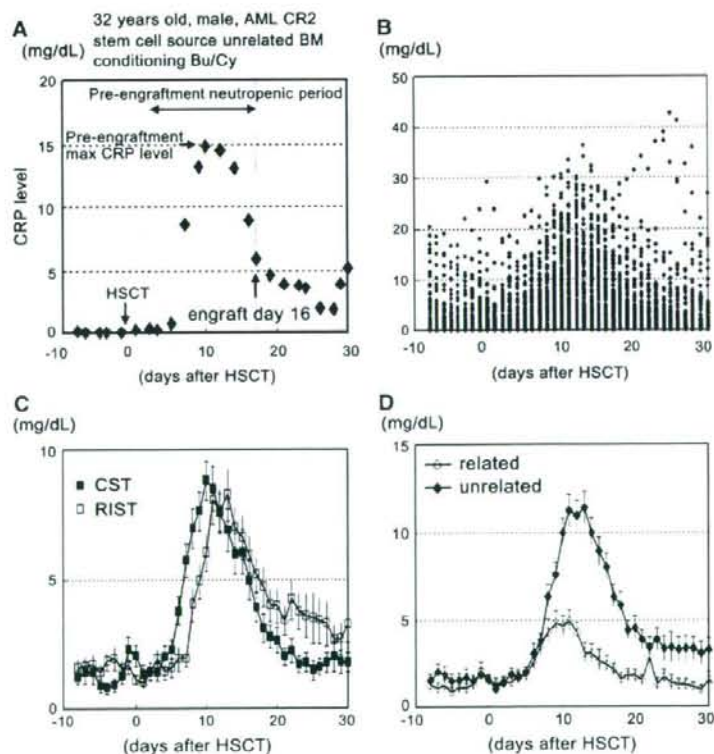


**Figure 1.** An example of how we measured CRP in a representative patient (A). Dot plot of the CRP level. All patients (B), CST versus RIST (C) and related versus unrelated (D).

statistics were used. Student *t*, chi-square, Fisher's exact test, and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. To analyze the pretransplant risk factors for a high CRP level, logistic analysis was used. OS was estimated using Kaplan-Meier curves. The cumulative incidence of aGVHD and NRM was estimated based on a Cox regression model for cause-specific hazards by treating progressive disease or relapse as a competing event. Cox proportional hazard models were used for the multivariate analysis of variables in aGVHD, NRM, and OS after HSCT. Clinical factors that were assessed for their association with aGVHD included patient age, patient sex, donor sex, CMV serostatus, conditioning regimen (CST versus RIST), donor (human leukocyte antigen [HLA]-matched versus HLA-mismatched, related versus unrelated), GVHD prophylaxis (cyclosporine-based versus tacrolimus-based, short-term MTX versus no MTX) and disease risk (standard versus high risk). NRM and OS were also assessed for their association with these factors. Factors with $P < .10$ in the univariate analyses were subjected to a multivariate analysis using a multiple logistic analysis and Cox proportional hazard modeling. In Japan, only BM and CB are allowed for unrelated transplantation, and most transplantations with a related donor use PBSC as a stem cell source. Therefore, the stem cell source was not included as a factor in the multivariate analysis. A level of $P < .05$ was defined as statistically significant. All *P* values are 2-sided. All analyses were made with SPSS ver 10.0 statistical software (Chicago, IL). This analysis was approved by the institutional review board.

## RESULTS

### Infections

The median duration of follow-up in surviving patients was 965 days (61 to 1432 days) in the high-CRP group and 915 days (76 to 1803 days) in the low-CRP group, and the incidence of total documented infections during neutropenia was, respectively, 23 cases in the high-CRP group (34%) and 27 cases in the low-CRP group (17%, $P = .004$). The incidence of bacteremia was, respectively, 20 cases (30%) and 20 cases (13%, $P = .002$), and the incidence of pneumonia was 7 cases (10%) and 4 cases (3%, $P = .01$). The incidence of central venous catheter infection was, respectively, 4 cases (6%) and 7 cases (4%, $P = .63$).

Serial changes in the CRP level are shown in Figure 1B; in most cases, the CRP level was elevated within 2 weeks of HSCT. Stratified data according to conditioning regimen (CST versus RIST) or relation to donor (related versus unrelated) are shown in Figure 1C and D, respectively.

To clarify the pretransplant risk factors for high CRP values during neutropenia, we performed a logistic regression analysis, which showed that male, unrelated donor, stem cell source with BM or CB transplantation (versus PBSCT), HLA-mismatched donor, and immunosuppression with MTX were associated with high CRP values during neutropenia (Table 1). Factors that showed significant associations (*P* < .1) were subjected to a multiple logistic regression analysis, and the results showed that unrelated donor, HLA mismatch and male sex were associated with high CRP ($P < .001$, $P = .005$, $P = .028$, respectively), as shown in Table 3. The median CRP levels after CST and RIST were 10.5 (0.3-31.3) and 6.2 (0.1-42.7), respectively, with a significant difference ($P = .017$) (Table 2). Notably, within the RIST group, the median CRP level was significantly lower in related than in unrelated transplantation (1.6 mg/dL [0.1-9.7] veruss 16.2 mg/dL [0.5-42.7]: $P < .001$). However, the logistic analysis failed to disclose any overall significant difference between CST and RIST.

### Primary Outcomes

The cumulative incidences of aGVHD grade II-IV and grade III-IV are shown, respectively, in Figure 2A and B. Grade II-IV and grade III-IV aGVHD were both more frequent in the high-CRP group than in the low-CRP group ($P = .001$ and $P = .04$, respectively). A Cox proportional hazard model showed that a high CRP level and CMV serostatus were associated with an increased risk of grade II-IV aGVHD (Table 4). Similar results were obtained when we included only the patients who received a myeloablative conditioning regimen (grade II-IV aGVHD 25% in the low-CRP group and 58% in the high-CRP group, $P < .001$, grade III-IV aGVHD 7% in the low-CRP group and 21% in the high-CRP group, $P = .047$).

### Secondary Outcomes

OS and NRM are shown, respectively, in Figure 3A and B. OS was significantly worse in the

**Table 3.** *Multiple Logistic Regression Analysis of Risk Factors for High CRP during Neutropenia*
*Factors with P < .10 in a Multivariate Analysis Was Shown**

| Outcomes and Variables | Multiple Logistic Regression Analysis | | |
|---|---|---|---|
| | Odds | 95% CI | P Value |
| Unrelated donor | 4.6 | 2.2-9.6 | <.001 |
| HLA mismatch | 2.6 | 1.3-5.0 | .005 |
| Patient sex (male) | 2.1 | 1.1-4.2 | .0028 |

CRP indicates C-reactive protein; CI, confidence interval; HLA, human leukocyte antigen; CMV, cytomegalovirus.

*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source.
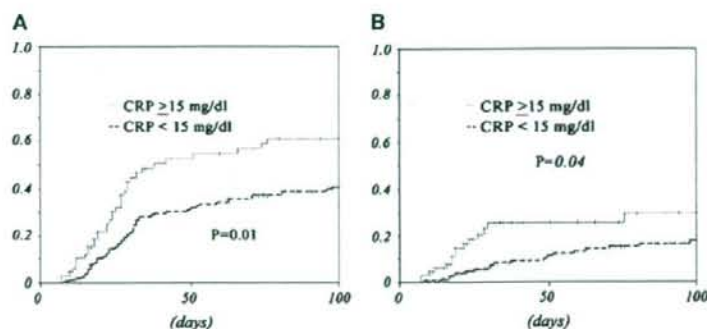
**Figure 2.** Cumulative incidence of grade II-IV aGVHD (A) and grade III-IV aGVHD (B) stratified according to the maximal CRP level during neutropenia.

high-CRP group than in the low-CRP group (1-year OS 47% versus 75%, $P = .001$). NRM was significantly higher in the high-CRP group than in the low-CRP group (1-year NRM 47% versus 13%, $P < .001$). Similar results were obtained when we included only patients who received a myeloablative conditioning regimen (1-year NRM 8% in the low-CRP group and 38% in the high-CRP group, $P = .007$). A Cox proportional hazard model showed that the risk factors for poor OS were high CRP ($P = .002$, hazard ratio [HR] 2.0, 95% confidence interval [CI] 1.3-3.1) and high-risk disease ($P = .015$, HR 2.2, 95% CI 1.2-4.0), whereas those for high NRM were high CRP ($P < .001$, HR 4.0, 95% CI 2.0-8.0) and high-risk disease ($P = .029$, HR 2.6, 95% CI 1.1-6.2), as shown in Table 4. When the threshold was set at 15 mg/dL, the sensitivity and specificity of the CRP level for prediction of grade II-IV aGVHD, NRM, or OS were 37% and 75%, 59% and 79%, and 40% and 78%, respectively. The relapse rate was significantly lower in the high-CRP group than in the low-CRP group (1-year relapse 21% versus 33%, $P = .02$).

Causes of death are summarized in Table 5. A total of 57 patients (36%) in the low-CRP group and 39 patients (58%) in the high-CRP group died ($P = .002$, OR 2.4 [1.4-4.4]). Six patients (4%) in the low- and 5 (7%) in the high-CRP group died because of aGVHD, for example, death because of infectious diseases associated with aGVHD and its treatment. Seven patients (4%) in the low- and 11 (16%) in the high-CRP group ($P = .003$, OR 4.2 [1.6-11.4]) died because of chronic GVHD (cGVHD), including death because of infectious diseases associated with cGVHD and its treatment. No patient (0%) in the low- and 5 (7%) in the high-CRP group ($P = .002$) died because of infectious diseases excluding infectious disease concomitant with GVHD. No patient in the low-CRP group and 4 (6%) in the high-CRP group ($P = .008$) died because of multiple-organ failure (MOF) excluding MOF because of GVHD and infectious disease.

## DISCUSSION

The results of this retrospective study suggested that higher CRP values during the neutropenic period may reflect net inflammation secondary to tissue damage because of the conditioning regimen, infection, and subsequent allogeneic immune reactions, all of which lead to aGVHD/cGVHD and ultimate NRM. In a mouse model, the concept that the production of inflammatory cytokines plays an important role in the development of aGVHD, by affecting the afferent and effector phase [12,13], has been accepted. Cooke et al. [26] showed that LPS antagonism reduced aGVHD in a mouse model, as indicated by Ferrara et al. [4]. However, in human studies, the value of determining individual levels of cytokines to monitor aGVHD has not been fully explored, because this approach is very costly and requires sophisticated techniques, which impedes its universal applicability. On the other hand, CRP is already being widely used

**Table 4.** *Multiple Variate Analysis for aGVHD, NRM, and OS**

| Outcomes and Variables | Hazard Ratio | 95% CI | P value |
|---|---|---|---|
| **Grade II-IV aGVHD** | | | |
| High CRP | 1.7 | 1.1-2.6 | .02 |
| CMV positivity | 3.1 | 1.0-9.8 | .5 |
| Disease risk (high) | 1.6 | 0.9-2.7 | .10 |
| **NRM** | | | |
| High CRP | 4.0 | 2.0-8.0 | <.001 |
| Age (≥40 years old) | 1.9 | 0.9-3.9 | .07 |
| Disease risk (high) | 2.6 | 1.1-6.2 | .03 |
| **OS** | | | |
| High CRP | 2.0 | 1.3-3.1 | .002 |
| Disease risk (high) | 2.2 | 1.2-4.0 | .02 |

CRP indicates C-reactive protein; CI, confidence interval; CMV, cytomegalovirus; GVHD, graft-versus -host disease; TBI, total body irradiation; NRM, nonrelapse mortality; OS, overall
*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source
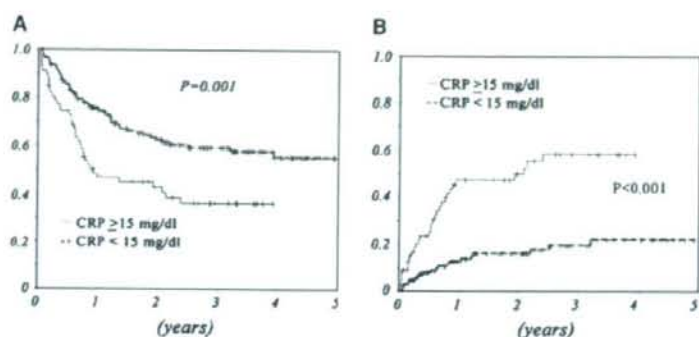
**Figure 3.** OS stratified according to the maximal CRP level during neutropenia (A). Cumulative incidence of TRM stratified according to the maximal CRP level during neutropenia (B).

worldwide, especially in Japan, to distinguish bacterial infections from other causes of fever [15-19]. Based on this practice, we reviewed the value of the CRP level after HSCT, and our data suggest that it might be useful to monitor the CRP value as a net surrogate marker for produced cytokines, and for predicting the subsequent development of aGVHD and NRM.

Our patients had various interacting backgrounds, and it is still difficult to predict whether a patient with a high CRP level is destined to suffer from GVHD or major infectious complications. Infectious diseases were previously reported to be a primary cause of elevated CRP [8,20], which might, in turn, affect the severity of aGVHD. In this study, we made every effort, including intense culture studies, to exclude infection as a primary cause of increased CRP, and showed that there were significantly more documented

infections in the high-CRP group than in the low-CRP group. Current practice for the prevention of infection mostly focuses on the effective control of Gram-negative bacteria, considering the potent immediate pathologic effect of the organisms. However, if the hypothesis that decreasing the net production of cytokines is important for the prevention of subsequent GVHD is correct, more effort should be paid to broadly cover other types of organisms or even clinically less significant infection, that is, stomatitis, at least during the early period of neutropenia, particularly in patients carrying risk factors for high CRP, which included unrelated donor, HLA mismatch, BM, and CB transplantation in this study. The addition of other markers, such as procalcitonin, may be useful for identifying the risk of major infectious complications [24].

**Table 5.** *Causes of Death Stratified According to CRP Value during Neutropenia*

| Causes of death | Low CRP Group CRP < 15 mg/dL n = 157 | High CRP Group CRP ≥ 15 mg/dL n = 67 | P Value |
|---|---|---|---|
| Total | 57 (36%) | 39 (58%) | .002 |
| Relapse/progressive disease | 34 (22%) | 8 (12%) | .09 |
| acute GVHD (total) | 6 (4%) | 5 (7%) | .25 |
| acute GVHD | 5 (3%) | 3 (5%) | .63 |
| acute GVHD + infection | 1 (1%) | 2 (3%) | .16 |
| chronic GVHD (total) | 7 (4%) | 11 (16%) | .003 |
| chronic GVHD | 3 (2%) | 7 (10%) | .005 |
| chronic GVHD + infection | 4 (3%) | 4 (6%) | .21 |
| Infection* | 0 (0%) | 5 (7%) | .002 |
| MOF† | 0 (0%) | 4 (6%) | .008 |
| Respiratory failure‡ | 3 (2%) | 4 (6%) | .11 |
| Others | Stroke 2 | VOD 1 | |
| | VOD 2 | Myocardial infarction 1 | |
| | Secondary cancer 1 | | |
| | Unknown 2 | | |

CRP indicates C-reactive protein; GVHD, graft-versus-host disease; TBI, total-body irradiation; MOF, multiple organ failure; VOD, veno-occlusive disease.
*Excluding infection during GVHD or GVHD treatment.
†Excluding MOF due to GVHD, infection.
‡Excluding respiratory failure because of GVHD, infection, and MOF.

Tissue damage caused by the conditioning regimen, complicated infections, and allogeneic immune reactions are the primary factors that are associated with the initial elevation of CRP early in the course of allogeneic HSCT. Consequently, it can be speculated that a reduced-intensity conditioning regimen results in decreased cytokine release and a resultant lower CRP value, which may lead to less chance of developing GVHD. Although the RIST regimens we used were relatively dose-intense, in this retrospective review we still found that CRP levels tended to be decreased after RIST compared to conventional myeloablative transplantation, particularly in a related compared to an unrelated transplantation setting. Because augmentation of allogeneic immune and inflammation reactions may induce a higher CRP value, we speculate that the benefit of RIST is diminished when a strong allogeneic reaction is induced, as in cases of unrelated transplantation.

To further evaluate the relationship between a higher CRP value during neutropenia and common risk factors associated with transplantation, we performed a multivariate analysis and showed that unrelated donor, HLA mismatch, and male sex were associated with higher CRP values. Additionally, from the finding in the multivariate analysis that unrelated donor and HLA mismatch were independently associated with high CRP, we surmised that the degree of genetic disparity might be associated with higher CRP during neutropenia. Based on a consideration of these findings together, we think that a higher CRP value may reflect the degree of tissue damage because of the transplant regimen and the subsequent magnitude of allogeneic immune reactions. Nevertheless, our analysis was hampered, because in Japan only BM and CB are allowed for unrelated transplantations, and most transplantations with a related donor use PBSC as a stem cell source. In these settings, a theoretically longer neutropenic period after unrelated BM or CB transplantation might be associated with a higher risk of infection, which could lead to higher CRP, as shown in this study.

In this study, the primary causes of death in the low-CRP group were mainly relapse and progression, whereas in the high-CRP group this was NRM. Notably, the observation that the relapse rate was higher in the low-CRP group than in the high-CRP group, as previously suggested by Min et al. [23], may further support our hypothesis that serum CRP values represent overall inflammation and cytokine production, which paves the way to GVHD and related graft-versus-leukemia (GVL) effects. A possible reason for this finding is that a low CRP level resulted in a lower incidence of GVHD and a resultant decrease in the GVL effect, or the high-CRP group developed earlier and more-frequent death from NRM compared to the low-CRP group, which left fewer patients for evaluation of the later occurrence of relapse.

In conclusion, our results suggest that the CRP value in the neutropenic period before engraftment in patients undergoing allogeneic HSCT may be a net surrogate marker of early inflammation that leads to the development of aGVHD/cGVHD and subsequent NRM, as has been proposed in mouse models. The intensity of the conditioning regimen, infectious diseases, and degree of allogeneic immune response attributed to HLA compatibility and the stem cell source may be the major factors that predict higher CRP values. Based on the results of this retrospective study, future clinical studies to evaluate the feasibility of earlier intervention and adjustment of the procedure for preventing GVHD and NRM based on monitoring of the early CRP value are warranted.

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## LETTER TO THE EDITOR

# Small intestinal CMV disease detected by capsule endoscopy after allogeneic hematopoietic SCT

CMV disease is a serious complication after allogeneic hematopoietic SCT (Allo-HSCT) in addition to GVHD.[1] CMV disease can involve many organs and the gastrointestinal tract is a common site.[2] There are several reports on small intestinal endoscopic findings of GVHD detected by capsule endoscopy,[3][6] but only limited information has been published regarding endoscopic findings of CMV enteritis after Allo-HSCT using capsule endoscopy.[4] We report herein a case of CMV enteritis involving the small intestine after Allo-HSCT that was detected by capsule endoscopy.

A 58-year-old man with myelodysplastic syndrome underwent Allo-HSCT with HLA mismatched unrelated cord blood at the National Cancer Center Hospital in Tokyo, Japan. The conditioning regimen consisted of fludarabine ($125 \, mg/m^2$), melphalan ($80 \, mg/m^2$) and 4 Gy TBI. Tacrolimus was administered for GVHD prophylaxis.

The transplantation course was uneventful for 7 months, but the patient then started experiencing epigastric pain and watery diarrhea. Total colonoscopy revealed several erosions surrounding a single ulceration in the ascending colon (Figure 1a). Biopsy specimens obtained from the ulceration and erosions showed enlarged endothelial cells with nuclear inclusion bodies (Figure 2a) that were positive for CMV by immunohistochemical staining (Figure 2b). No definite histological feature to support GVHD was found. A simultaneous CMV antigenemia assay using the monoclonal antibody C7-HRP (Teijin, Tokyo, Japan) indicated two positive cells per 44 000 cells. A subsequent

capsule endoscopy (PillCam SB, Given Imaging Inc., Israel) also revealed a single ulceration with satellite erosions in the jejunum, but we were unable to perform a biopsy because of the primary limitation of capsule endoscopy, that is, lack of any biopsy capability. The ulceration in the jejunum was very similar to the one found in the ascending colon (Figure 1b).

Antiviral therapy was started with ganciclovir (10 mg/kg/day) followed by foscarnet (60 mg/kg/day) for 6 weeks and the patient's symptoms resolved completely. A follow-up CMV antigenemia assay was negative and endoscopic findings by capsule endoscopy and total colonoscopy revealed healing scars without any active lesions.

Intestinal complications after Allo-HSCT predominantly affect the small intestine,[7] but macroscopic findings of small intestinal disorders following Allo-HSCT have not been fully investigated. Traditional small bowel examinations such as push enteroscopy are somewhat invasive in nature and most patients undergoing Allo-HSCT cannot tolerate such procedures due to the seriousness of their condition. Capsule endoscopy is now widely accepted for small intestinal investigation as being far less invasive. In the present case, this advanced technology enabled us to obtain clear endoscopic images of CMV enteritis in the small intestine after Allo-HSCT.

CMV antigenemia assay is one of the most widely used methods to detect CMV reactivation in a variety of clinical settings;[8] however, it is of little value in predicting and diagnosing gastrointestinal CMV disease.[9] The differential diagnosis of intestinal disorders following Allo-HSCT includes intestinal GVHD, thrombotic microangiopathy, treatment-related toxicities and *clostridium difficile*
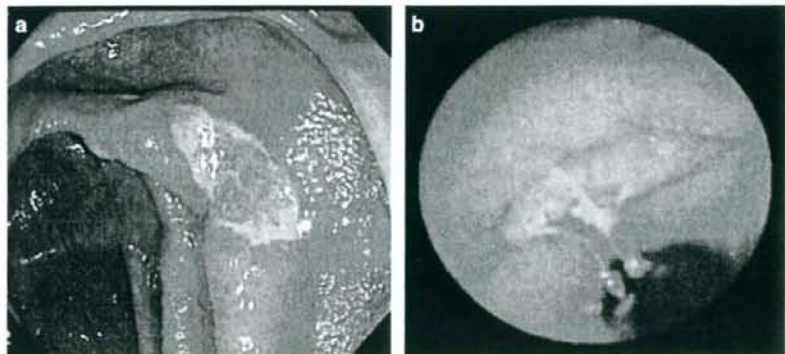


**Figure 1** Total colonoscopy revealed a single ulceration (a) with several erosions in the ascending colon. Capsule endoscopy also revealed a single ulceration (b) with several erosions in the jejunum very similar to the one found in the ascending colon.
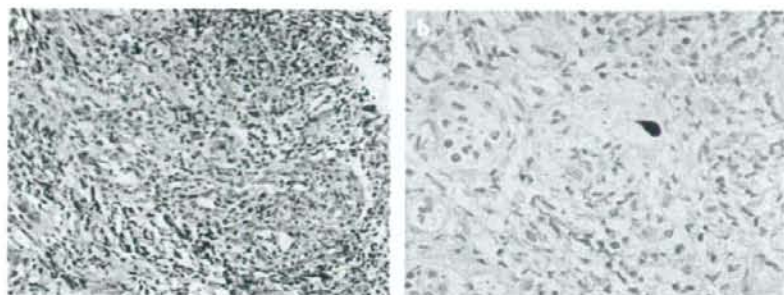
**Figure 2** Biopsy specimens obtained from the ulceration and erosions showed cellular enlargement of endothelial cells with nuclear inclusion bodies (a) that were positive for CMV by immunohistochemical staining (b).

enterocolitis as well as CMV enterocolitis.[7] Actual diagnosis is usually based on pathological examinations of endoscopically obtained mucosal biopsy specimens, but this is not possible with capsule endoscopy due to its lack of biopsy capability.

In the present case, we were able to make a diagnosis of CMV enteritis in the small bowel without histological biopsy because of similar coincident findings in the ascending colon that were proven by pathological examination to be CMV colitis. This diagnosis was subsequently confirmed clinically by prompt resolution of intestinal symptoms, endoscopic findings and CMV antigenemia assay after antiviral treatment using ganciclovir and foscarnet.

Fortunately, CMV enteritis involved both the small intestine and colon in this case. CMV colitis, which was proven by biopsy, was a factor in diagnosing small intestinal CMV disease; however, CMV enteritis involving only the small intestine without colon involvement may not be rare after Allo-HSCT because the small intestine is a frequent and severe site for gastrointestinal complications following Allo-HSCT.[7] In such a situation, our picture (Figure 1a) showing ulceration detected by capsule endoscopy may be useful in diagnosing small intestinal CMV disease after Allo-HSCT.

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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 ($22/\mu l$) and those who did not ($12/\mu l$). Serial tetramer analysis showed that 21% of the patients had $>10/\mu l$ CMV-specific CTL at the first detection of CMV antigenemia and 67% of the patients had more than $10/\mu l$ 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-$\gamma$ producing CD4+ and CD8+ lymphocytes were suppressed at the onset of CMV colitis (1.6 and $8/\mu l$), which increased with recovery of the disease (19 and $47/\mu l$). 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 immuno-monitoring against CMV.
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**Keywords:** CMV; intracellular IFN-$\gamma$; 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 $>10–20/\mu l$ 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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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 $30 \pm 7$ and $60 \pm 7$ 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.[15] 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
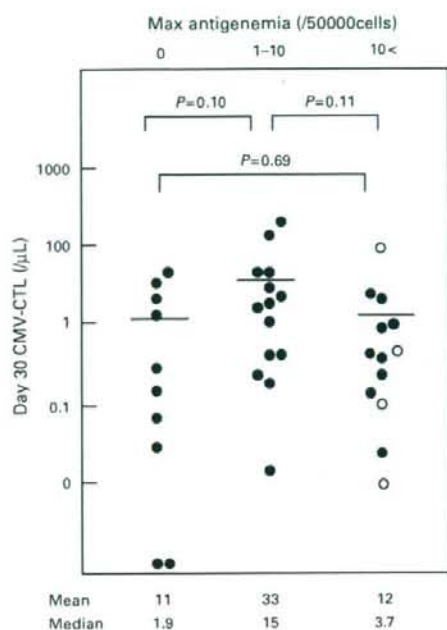
**Figure 1** The number of CMV-specific CTL as evaluated by tetramer assay on day 30 post transplantation. The number of CMV-specific CTL did not differ between patients who did not develop CMV antigenemia, who had antigenemia below 10/50 000, who had antigenemia of >10/50 000. The outlined circle ○ 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*
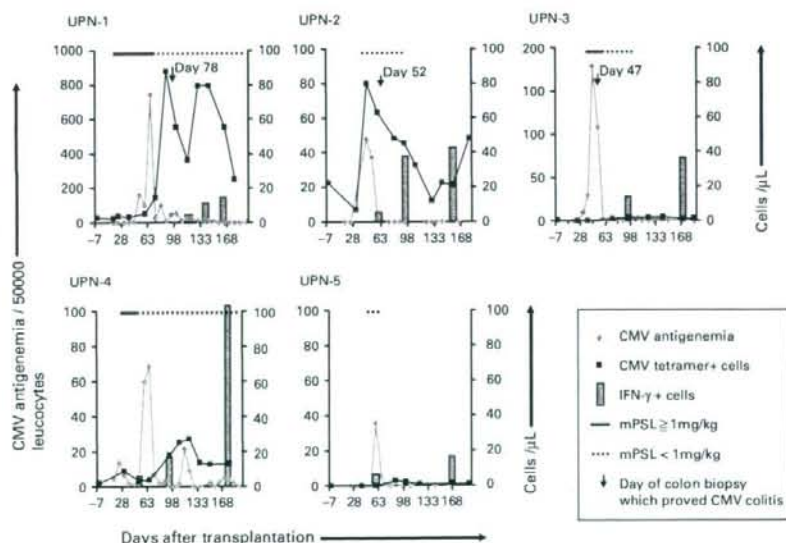Upon stimulation with CMV lysate, intracellular IFN-γ staining among five patients (UPN1–5) who developed high



**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.