TABLE 1. Characteristics of 64 patients.

Sex (Male/Female)	38/26
Median age in years (range)	47 (17 - 68)
Underlying disease	
Acute nonlymphoblastic leukemia	16
Acute lymphoblastic leukemia	8
Myelodysplastic syndrome	7
Malignant lymphoma	26
Agnogenic myeloid metaplasia	1
Plasma cell neoplasm	2
Solid tumor	4
Conditioning regimen	
Cyclophosphamide/TBI 12 Gy	6
Busulfan/Cyclophosphamide	10
Fludarabine/Busulfan	15
Fludarabine/Busulfan/TBI 4 Gy	18
Fludarabine/Busulfan/ATG	1
Fludarabine/Melphalan/TBI 4 Gy	1
Cladribine/Busulfan	8
Cladribine/Busulfan/TBI 4 Gy	2
Cladribine/Busulfan/ATG	2
Other	1
Type of donor	
HLA-identical sibling	32
HLA-mismatched family	4
Unrelated donor	28
Type of graft	
Bone Marrow	18
Blood stem cells	34
Cord blood stem cells	12
GVHD prophylaxis	
Cyclosporin A	23
Cyclosporin A/Methotrexate	38
Tacrolimus	3
CMV serostatus (recipient/donor)	
+/+	40
+/-	9
+/ ND	10
-/+	3
-/-	2

*TBI, total body irradiation; ATG, anti-thymocyte globulin;
GVHD, graft-versus-host disease; CMV, cytomegalovirus; ND, no data.

infection after reinduction chemotherapy with idarubicin and cytosine arabinoside. Cyclosporine A alone, Cyclosporine A with methotrexate or tacrolimus alone, orally or intravenously was given to prevent GVHD. Ciprofloxacin, fluconazole, and sulfamethoxazole/ trimethoprim were given as prophylaxis for bacterial, fungal, and pneumocystis carinii infections. All patients were given acyclovir 750 mg/day orally from day -7 to the discontinuation of GVHD prophylaxis.

CMV antigenemia assay

CMV antigenemia assay was performed by the method described previously (van der Bij et al. 1988; Gondo et al. 1994). In brief, 150,000 peripheral blood leukocytes were cytocentrifuged on a slide and fixed with acetone within 6 hours after specimen collection. The cells were incubated with monoclonal antibody horseradish peroxidase-C7 (Teijin, Tokyo) raised against the pp65 antigen of CMV, and stained by the direct immunoperoxydase method. Under light microscopy, CMV antigen-positive cells were enumerated. The degree of antigenemia was expressed as the number of positively stained cells per 50,000 leukocytes.

Amplification and detection of viral DNA

DNA was extracted from 200 μl of blood plasma with a MagNA Pure instrument (Roche Molecular Biochemicals, Mannheim, Germany). A MagNA Pure LC DNA Isolation Kit I was used according to the manufacturer's instructions. In the initial 42 samples, DNA was extracted manually using a QIAmp Blood mini-kit (Qiagen, Valencia, CA, USA).

We selected and designed the sequences of primers in the UL75 region of CMV. The forward and reverse primers of UL75 were 5′-CCT TGC GTG TCG TCG TAT TCT AGC-3′, and 5′-GCC TCA TCA TCA CCC AAA CGG ACA G-3′, respectively. PCR amplification was performed in a total volume of 20 μl in the presence of 2 μl of DNA sample, 4.4 μl of PCR master mix (Takara Ex Taq R-PCR Version, Takara, Shiga), 10 pmol of each of the primers, 2 mmol of $MgCl_2$, 2 μl (1 : 15,000 dilution) of Syber Green I (SYBR Green I Nucleic Acid Gel Stains, Takara), and distilled water.

PCR was performed with a LightCycler instrument using the SYBR Green I system (Roche Diagnostics, Tokyo) under the following conditions: 95°C for 30 sec and 35 cycles at 95°C for 0 sec (i.e., an instrument setting of zero seconds), 57°C for 5 sec, and 72°C for 10 sec. Crossing points were determined by the operator dependent fit points method. Melting curve analysis was performed on all of the positive results. The melting temperature range used to determine that a specimen yielding a crossing point was a true positive was 86.5 to 87.5°C.

Positive control and negative control

Positive PCR product DNA from a clinical sample was confirmed based on its positivity in electrophoresis and selected as the PCR standard. A direct sequence analysis was performed on this PCR product, which was confirmed to show a 100% match with GenBank data (NC_001347, similar as AD169 strain). The DNA from this sample was then subjected to molecular cloning by being inserted into the pCR4-TOPO vector (TOPO cloning kit, Invitrogen, Carlsbad, CA, USA). This plasmid was used as the CMV standard in this study. CMV was quantified using serially diluted CMV standards within the range of 1.41×10 to 1.41×10^8 copies per μl, and the numbers of CMV copies were calculated. The specificity of the CMV PCR was assessed by testing murine CMV (Smith strain) and herpes simplex viruses (HSV) (HSV-1; KOS strain and HSV-2; Savage strain) as negative controls. The CMV PCR assay with 35 cycles was negative for these samples.

Preemptive therapy for prevention of CMV disease

The decision to start preemptive therapy was based on the detection of 10 or more positive cells per 50,000 cells by the antigenemia assay, or by the clinical diagnosis of CMV disease with apparent or infection-related symptoms. In this study, PCR results were not used in the decision-making process regarding preemptive therapy. Intravenous ganciclovir was started at 5 mg/kg/day three times per week, and was continued until the positive cell count had decreased to less than 10 per 50,000 cells. If the number of positive cells increased or CMV-related symptoms worsened, the dose of ganciclovir was increased.

Statistical analysis and definition of CMV infection

Correlations between the number of CMV DNA and CMV antigen-positive cells were analyzed by calculating Spearman ranked correlation coefficient. Differences between two groups were assessed by the Mann-Whitney's U-test. ROC (Receiver Operating Characteristic) analysis was performed using ROCKIT 0.9B (Dorfman et al. 1992). The diagnostic power of UL75-PCR was assessed by calculating of the Area

under the ROC Curve (AUC). We accessed several cut-off values for positive cells, 1, 5, 10, or 50 per 50,000 examined cells, in the antigenemia assay to reflect "positive for CMV infection" on the ROC analysis. *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinical sample amplification

Threshold cycle values of the serially diluted standard were plotted (Fig. 1A), and showed good linearity (Fig. 1B). The amplification results of

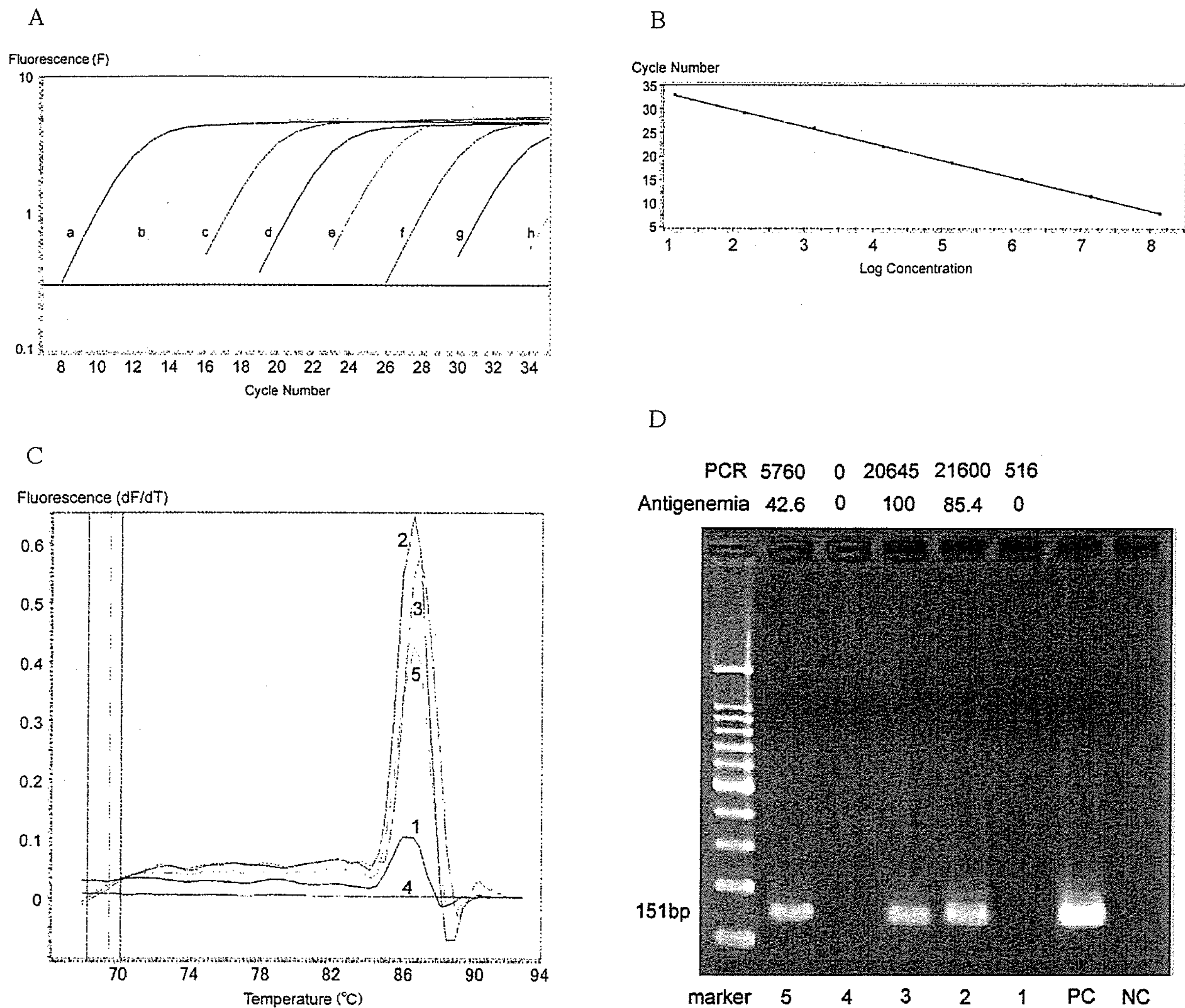

Fig. 1. Real-time PCR for CMV.
(A) Amplification plots obtained with the control plasmid for CMV. Serial 10-fold dilutions with 1.41×10^8 (a) to 1.41×10 (h) copies per reaction were amplified for 35 cycles. The horizontal line represents threshold of fluorescence signal. (B) Crossing points (cycle number) plotted against the log of copy number to obtain a calibration curve. The assay was linear from 1.41×10 to 1.41×10^8 copies per μl. (C) Melting curves for PCR products of five randomly selected clinical samples are shown. No extra peak was observed. (D) Amplification of PCR was confirmed by 2% agarose gel electrophoresis, which showed tight single bands, except in lanes 1 and 4. The numbers in upper line above the gel indicate the copy numbers per ml in cases 1, 2, 3, 4 and 5. The numbers in lower line above the gel indicate the corresponding results of the antigenemia test per 50,000 examined cells. Panels (C) and (D) share the same case numbers. The expected size of the PCR product is 151 bp. PC, positive control; NC, negative control.

five randomly selected clinical samples are shown as representative data. To confirm the purity of amplification, melting curves for PCR products after amplification were examined and the presence of an extra peak was neglected (Fig. 1C). Furthermore, amplification was confirmed by 2% agarose gel electrophoresis. Three of five samples showed a tight single 151 base-pair band in each lane, except lanes 1 and 4 (Fig. 1D), indicating that only one product was amplified. As a reference, the calculated copy numbers and the results of the antigenemia test for these samples are shown in Fig. 1D.

Comparison of the pp65 antigenemia assay with CMV real-time PCR

A total of 357 weekly samples were obtained from 64 patients and analyzed by both real-time PCR and antigenemia assays. As a result, a total of 158 samples obtained from 40 patients were positive for antigenemia assay, 220 samples from 55 patients were positive for real-time PCR which were confirmed by electrophoresis and melting curve analysis, 113 samples were negative for both assays, and 134 were positive for both assays (Kappa = 0.53). Eighty-six samples were positive by real-time PCR and negative by the antigenemia assay, while 24 samples were negative by real-time PCR and positive by the antigenemia assay (Table 2). The median number of pp65-positive cells in these 24 samples was 2 (range, 1.4 – 10.3) per 50,000 leukocytes.

There was a correlation between the CMV DNA copy number and the pp65-positive cell count (R = 0.738, $p < 0.0001$ by the Spearman test) (Fig. 2). Samples from patients were classified into four groups according to the results of the antigenemia assay (Fig. 3). Group 1 ($n = 199$) was negative by the antigenemia assay, group 2 ($n = 75$) was positive at 1 to 9 cells, group 3 ($n = 42$) was positive at 10 to 49 cells, and group 4 ($n = 41$) was positive at > 50 cells. The median CMV DNA load was 0 copies/ml (range, 0 to 39,345) in group 1, 344 (2.5 $\log_{10}$) copies/ml (0 to 172, 750) in group 2, 4,910 (3.7 $\log_{10}$) copies/ml (0 to 141,650) in group 3, and 22,200 (4.3 $\log_{10}$) copies/ml (2,285 to 464,750) in group 4. The CMV DNA load was significantly different in each group, as shown in Fig. 3 ($p < 0.0001$ by the Kuruskal-Wallis test). The specimens negative by real-time PCR or the antigenemia test are not depicted on the graphs in Fig. 2 and 3.

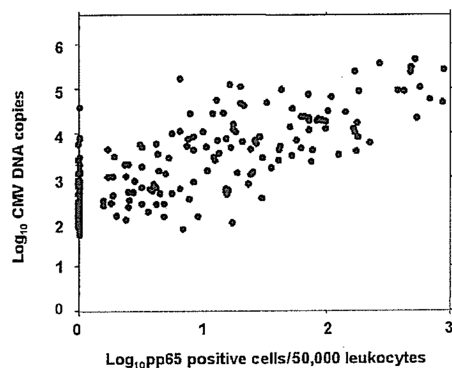

Fig. 2. Correlation between CMV DNA copy number in plasma and CMV pp65-positive cells. The CMV copy number was plotted on a logarithmic graph against the number of pp65-positive cells. The correlation between the two tests was examined (R = 0.738, $p < 0.0001$ by the Spearman test). The specimens negative by real-time PCR or the antigenemia test are not depicted. Minimum value by real-time PCR was 52 copies/ml.

TABLE 2. Results of real-time CMV PCR and CMV pp65 antigenemia of blood plasma for 357 samples.

CMV pp65 antigenemia	CMV PCR in blood plasma		Total
	Positive	Negative	
Positive	134	24	158
Negative	86	113	199
Total	220	137	357

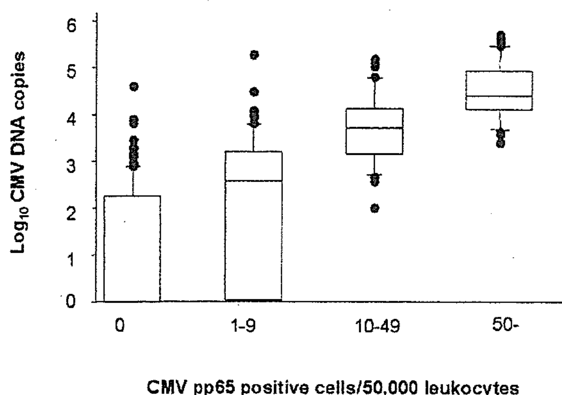

Fig. 3. CMV DNA loads in plasma based on the number of CMV pp65-positive cells per 50,000 leukocytes. Samples were classified into four groups: group 1 (n = 199), negative by the antigenemia assay; group 2 (n = 75), 1 to 9 positive cells; group 3 (n = 42), 10 to 49; and group 4 (n = 41), 50 and more. The median CMV DNA levels in samples were 0 copies/ml (range, 0 to 39,345), 344 (2.5 $\log_{10}$) copies/ml (range, 0 to 172,750), 4910 (3.7 $\log_{10}$) copies/ml (0 to 141,650), and 22,200 (4.3 $\log_{10}$) copies/ml (2,285 to 464,750), respectively. The CMV DNA load was significantly different among the four groups ($p < 0.0001$ by the Kuruskal-Wallis test). The box-and-whisker plots show 10th, 25th, 50th, 75th, 90th percentile values. The specimens negative by real-time PCR are not depicted. Outliers are indicated by dots.

Longitudinal analysis of the first detection of CMV DNA and CMV antigenemia cells

The CMV PCR and CMV antigenemia assays became positive simultaneously in 13 of 64 patients. The first positive PCR test preceded the first positive antigenemia by 14 days (7 to 35) in 20 patients, while the first positive antigenemia preceded the first positive PCR by 7 days in 4 patients. CMV PCR alone was positive in 18 patients, and CMV antigenemia alone was positive in 3 patients. Neither CMV PCR nor CMV antigenemia was positive in 6 patients. The median number of days for the first development of positive antigenemia and PCR results after transplantation was 34 days (12 to 141) and 20 days (12 to 97), respectively. Therefore, CMV PCR was positive significantly earlier than the CMV antigenemia assay ($p < 0.0001$ by the Mann Whitney's U-test). The WBC count at the first development of positive PCR was significantly lower, with a median of 2,700/μl (range, 100 to 27,400) than at the first positive antigenemia, with a median of 4,700/μl (700 to 40,900, $p = 0.027$ by Mann Whitney's U-test). In 23 of 64 patients, preemptive ganciclovir therapy was started upon the detection of 10 or more positive cells per 50,000 cells by the antigenemia assay at a median of day 39 (range, 18 to 122) after transplantation. Despite the preemptive therapy, five of 23 patients developed CMV disease at a median of day 50 (38 to 123) after transplantation. In five of 64 patients, ganciclovir was started based on the clinical diagnosis of CMV disease with apparent or infection-related symptoms at a median of day 38 (34 to 42).

Incidence of CMV disease

Ten of the 64 patients developed CMV disease. The time pattern of PCR and antigenemia detection relative to the onset of CMV disease in the 10 patients is shown in Fig. 4. Eight patients had CMV colitis, one had CMV retinitis (patient no. 8 in Fig. 4), and one had CMV pneumonia (patient no. 3). In these 10 cases, cord blood transplants were performed in three cases, transplants from an HLA-mismatched unrelated donor were performed in two, transplant from an HLA-matched unrelated was performed in one, transplant from an HLA-mismatched sibling was performed in one, and transplants from an HLA-identical sibling were performed in three. The median number of days to the onset of CMV disease was 42 days after transplant (33 to 123 days). CMV reactivation was detected 3 days or more before the development of CMV disease in 8 of 10 patients by real-time PCR. However, CMV antigenemia detected CMV reactivation earlier than the development of CMV disease only in 4 of 10 patients (Fig. 4). Five patients with CMV disease were successfully treated with ganciclovir and are currently alive, but the other five patients with CMV disease died from causes other than CMV disease. None of the patients in this series died of causes directly related to CMV disease.

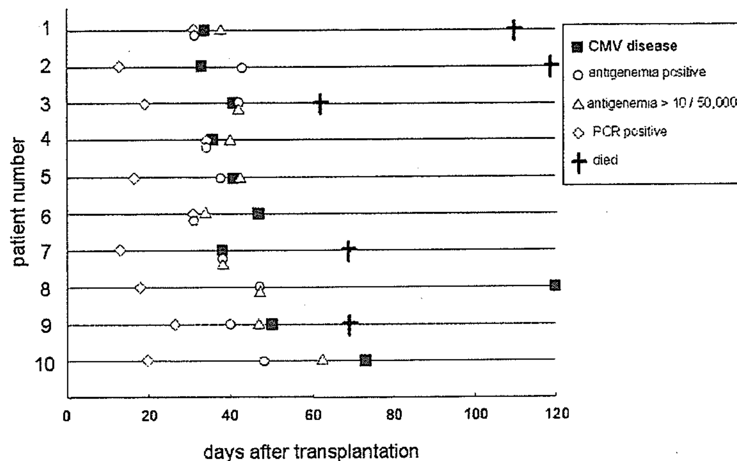

Fig. 4. Clinical course of individual patients with CMV disease. Solid squares, onset of CMV disease; open circles, first positive antigenemia; open triangles, first detection of > 10 positive cells per 50,000 cells by the antigenemia assay; open diamonds, first positive real-time PCR; cross, date of death.

The five deaths were caused by acute GVHD in one, disease progression in one, fungal infection in one, and bacterial infection in two. The peak CMV DNA load was significantly higher in patients with CMV disease, with a median of 82,250 copies/ml (range, 1,468 to 464,750) than in those without CMV disease, with a median of 628 copies/ml (0 to 374,150, $p = 0.005$, by Wilcoxon test).

Receiver Operating Characteristic (ROC) curve analysis

The ROC curves for UL75 PCR are shown in Fig. 5, in which four cut-off points for positive antigenemia, 1, 5, 10, or 50 positive cells per 50,000, were used. The LightCycler assay was clearly better than the discrimination limit. The Area Under the ROC Curve (AUC) values were 0.8426, 0.9240, 0.9483, and 0.9564, respectively, when 1, 5, 10, and 50 positive cells was used as a cut-off point for positive antigenemia. The 95% confidence intervals were as follows: 0.7917 – 0.8846, 0.8831 – 0.9529, 0.9205 – 0.9677, and 0.9311 – 0.9736, respectively. When the cut-off point was based on 10 pp65-positive cells per 50,000 cells, there was a better correlation between the CMV antigenemia assay and CMV PCR than when the cut-off point was based on 1

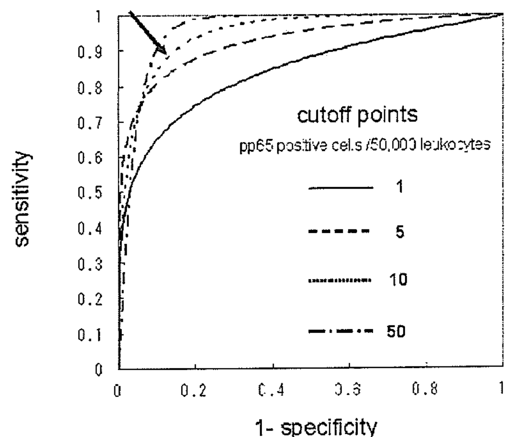

Fig. 5. ROC curves for UL75 PCR, with four cut-off points for the positivity for antigenemia: 1, 5, 10, or 50 cells per 50,000. The assay was far better than the discrimination limit. The AUC (Area Under the ROC Curve) values are 0.8426, 0.9240, 0.9483, and 0.9564, respectively. The 95% confidence intervals are as follows: 0.7917 – 0.8846, 0.8831 – 0.9529, 0.9205 – 0.9677, and 0.9311 – 0.9736, respectively. A tentative cut-off point at copy number 1,600 (arrow) gave a sensitivity of 87.2% and a specificity of 87.9% on the ROC curve in which 10 pp65-positive cells was used as the threshold for positive antigenemia.

pp65-positive cell. The cut-off points based on 5, 10, and 50 pp65-positive cells did not significantly differ from each other by the ROC analysis. A tentative cut-off point at copy number 1,600, as indicated by the arrow in Fig. 5, gave a sensitivity of 87.2% and specificity of 87.9% on the ROC curve, where 10 pp65-positive cells was used as the threshold for positive antigenemia.

Discussion

In our study, the CMV DNA copy number determined by real-time CMV PCR using SYBR Green I correlated with the number of pp65-positive cells, in agreement with the results of other studies using a TaqMan-based assay (Gault et al. 2001; Sanchez et al. 2001; Leruez-Ville et al. 2003; Li et al. 2003; Ikewaki et al. 2005). ROC analysis showed that this real-time CMV PCR exhibited adequate sensitivity and specificity, using the pp65 antigenemia assay as a reference standard. Corresponding to a pp65 antigenemia value of 10 positive cells per 50,000 leukocytes, which is used as the cutoff point for initiating preemptive therapy at our institute, a CMV DNA load of 1,600 copies/ml will be proposed as a cutoff point. However, we might have to start preemptive ganciclovir therapy at a cut off value of less than 1,600 copies/ml for high-risk patients. Our real-time PCR detected CMV-DNA earlier than the antigenemia assay detected CMV reactivation in the patients with CMV disease. Our real-time PCR makes it possible to adjust timing to start preemptive therapy more accurately according to the risk for CMV disease. The risks for CMV infection and/or disease have been increasing as new strategies have been introduced in allo-HSCT, including reduced-intensity stem cell transplantation, which enables older and/or heavily pretreated patients to undergo allo-HSCT, and HLA-mismatched transplantation with T-cell depletion or anti-T-cell agents (Kanda et al. 2001a; Nakai et al. 2002). Furthermore, Tomonari et al. (2003) suggested that the recovery of CMV-specific immunity after cord blood transplantation is delayed compared to that after BMT. Hence, in highly immunocompromised recipients, it may be advisable to set a lower cutoff point for the CMV DNA load to prevent CMV disease at the earliest stage. In this study, 47 patients underwent reduced-intensity stem cell transplantations (RIST) and 16 patients underwent conventional myeloablative stem cell transplantations (CST). The incidence of positive CMV antigenemia in the RIST group (33 of 47 patients, 70%) was higher than in the CST group (6 of 16 patients, 38%, $p = 0.019$). However, there was no significant difference in the incidence of positive PCR (85% and 88%) and CMV disease (17% and 13%) between the RIST group and the CST group. A higher incidence of CMV disease (10 of 64 patients, 16%) was observed and this might be attributed to our preemptive protocol consisting of a higher cutoff point and a lower initial dose of ganciclovir. It has been reported that preemptive therapy based on CMV antigenemia let to the more frequent development of CMV diseases other than pneumonitits, including gastrointestinal (GI) diseases, retinitis, and hepatitis (Boeckh et al. 1996; Kanda et al. 2001b). Mori et al. (2004) reported that among 19 and 14 patients who developed CMV-GI disease, only 4 (21%) and 7 patients (50%), respectively, became positive for antigenemia and plasma real-time PCR before the onset of CMV-GI disease. On the other hand, our plasma real-time PCR could detect CMV-DNA before the development of devastating CMV diseases in eight of 10 patients. The immediate initiation of antiviral therapy at low CMV DNA levels may become critical for highly immunocompromised recipients.

The detection of PCR products by the DNA-intercalating dye SYBR Green I system is simpler, cheaper, and probably more sensitive, since many fluorescent labels, rather than a single molecule, are incorporated into the amplified fragment (Karsai et al. 2002). However, this technique is not sequence-specific, and consequently nonspecifically amplified PCR products and primer dimers will also be detected. Nevertheless, we performed a melting curve analysis and electrophoresis to confirm that specific PCR products were formed, and these demonstrated specific and reproducible results.

We chose plasma as an assay material since

it still can be used during neutropenia (Boeckh et al. 1997). Plasma does not require labor-intensive processing such as isolation, counting, and adjustment of the number of peripheral blood leukocytes (PBL) before final analysis. It does not necessarily require normalization by a housekeeping gene, which is needed in real-time PCR using whole blood or PBL (Gault et al. 2001; Li et al. 2003). Despite these advantages of plasma as an assay material, it was long considered to be a poor source for PCR-driven assays because of its low sensitivity. Since the presence of CMV is strongly associated with cell components, it is considered that samples that incorporate whole blood or PBL provide a more sensitive detection of virus than plasma (Boeckh et al. 1997). However, recent improvements in plasma-based PCR assay have provided high sensitivity and clinical usefulness (Boeckh et al. 2004; Kalpoe et al. 2004; Schvoerer et al. 2005). Leruez-Ville et al. (2003) reported that a smaller PCR target (74bp) could provide better quantitative results because CMV DNA of plasma might be highly fragmented. In their study, the sensitivity of the PCR test was equivalent for plasma and whole blood. Although we did not compare the sensitivities with different components of blood, we demonstrated that our plasma real-time PCR was more sensitive than CMV antigenemia, perhaps due to the use of SYBR green I, i.e., the PCR target used in this study was 151 bp, which is not small enough to explain the improved sensitivity.

DNA extraction could strongly affect the assay's reproducibility (Gault et al. 2001). Mengelle et al. (2003) demonstrated that automated extraction and quantification of DNA from whole blood, instead of separated and counted PBL, provided acceptable results. They reported that 3.4 $\log_{10}$ genome copies in 200 μl of whole blood was equivalent to a threshold value of 50 pp65-positive cells per 200,000 cells. We applied the automated extraction of DNA in plasma and demonstrated that the plasma sample had an equivalent sensitivity for preemptive therapy for CMV. Hong et al. (2004) also reported that the combination of automated plasma DNA preparation and real-time PCR detection allowed for a sensitive assay of CMV viral load after bone marrow transplantation. It is evident that the standardization of plasma separation and storage methods should improve the reproducibility of plasma CMV DNA assays, which may further support the value of automated DNA extraction.

Our real-time PCR system for the measurement of CMV DNA in plasma using automated extraction and SYBR Green I dye appears to be a practical and simple system for obtaining reliable data and early detection of CMV reactivation, particularly for the purpose of prospectively guiding preemptive therapy for CMV disease after allo-HSCT.

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Case Report

Successful Treatment of Minimal Residual Disease–Positive Philadelphia Chromosome–Positive Acute Lymphoblastic Leukemia with Imatinib Followed by Reduced-Intensity Unrelated Cord Blood Transplantation after Allogeneic Peripheral Blood Stem Cell Transplantation

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Abstract

We describe a 35-year-old woman with Philadelphia chromosome–positive acute lymphoblastic leukemia (Ph⁺ ALL) who received allogeneic sibling donor peripheral blood stem cell transplantation (PBSCT) and entered a second complete remission. Upon detection of BCR-ABL transcripts after PBSCT, the patient received imatinib, leading to molecular remission. Following the failure of donor leukocyte infusions, she underwent reduced-intensity unrelated cord blood transplantation (RI-UCBT), and has continued durable molecular remission for more than 30 months without substantial graft-versus-host disease. Because of a lack of adverse effects of imatinib on transplantation outcome, a treatment strategy consisting of molecular monitoring–guided initiation of imatinib followed by RI-UCBT may be promising in the management of Ph⁺ ALL after allogeneic SCT.

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Key words: Philadelphia chromosome–positive acute lymphoblastic leukemia; Unrelated cord blood transplantation; Imatinib; Minimal residual disease

1. Introduction

The prognosis for adult patients with Philadelphia chromosome–positive acute lymphoblastic leukemia (Ph⁺ ALL) is poor. Although allogeneic stem cell transplantation (SCT) is considered the only potentially curative therapy, a substantial proportion of patients undergoing allogeneic SCT develop hematologic relapse or experience disease progression. In such cases, further treatment is rarely successful [1-5]. Consequently, identification of patients at the highest risk prior to overt hematologic relapse is of great importance. The reverse transcription polymerase chain reaction (RT-PCR) is a sensitive method for detecting low-level transcripts of the breakpoint cluster region–Abelson oncogene locus (BCR-ABL) to assess minimal residual disease (MRD) in Ph⁺ ALL [6]. Detection of BCR-ABL transcripts after allogeneic SCT is associated with a probability of hematologic relapse exceeding 90% [6,7].

Another important goal is to prevent MRD after allogeneic SCT from developing hematologic relapse. Imatinib (Glivec, STI571; Novartis Pharmaceuticals, East Hanover, NJ, USA), a selective protein tyrosine kinase inhibitor of BCR-ABL, has pronounced but brief antileukemic activity in patients with advanced Ph⁺ ALL, including those with failing SCT [4,5]. Patients with Ph⁺ ALL receiving imatinib after SCT on the basis of BCR-ABL transcript positivity have been shown to have a decreased rate of hematologic relapse and to experience prolonged disease-free survival (DFS) [8]. However, sustained molecular remissions are almost never expected with single-agent imatinib [4,5,8]. Despite the need

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for additional treatment options, no effective therapies are available at present.

Here, we report a Ph⁺ ALL patient with molecular evidence of recurrent leukemia after allogeneic peripheral blood stem cell transplantation (PBSCT) who was successfully treated with an unrelated cord blood transplantation (UCBT) following the induction of molecular remission by imatinib.

2. Case Report

In June 2002, a 35-year-old woman received a diagnosis of Ph⁺ ALL with additional karyotype abnormalities of –7 and der(9;22)(q10;q10). Major BCR-ABL chimeric messenger RNA was detected by RT-PCR. Although the patient achieved complete remission (CR) with an induction therapy consisting of cyclophosphamide, daunorubicin, vincristine, prednisolone, and L-asparaginase, based on the Japan Adult Leukemia Study Group ALL-97 protocol, she underwent hematologic relapse during the first course of consolidation therapy. The patient achieved a second CR, a complete cytogenetic remission but not molecular remission, in October 2002 after receiving imatinib at a daily oral dose of 600 mg for 27 days combined with vincristine and prednisolone. Immediately following conditioning therapy with cyclophosphamide at 120 mg/kg, cytarabine at 8 g/m^2, and total body irradiation with 12 Gy, the patient underwent an HLA-identical PBSCT (17.7 $\times$ 10^6/kg body weight CD34⁺ cells) with a brother as the stem cell donor. Cyclosporine A (CSA) and short-term methotrexate were used for prophylaxis against graft-versus-host disease (GVHD). The patient developed acute GVHD with stage 1 liver damage on day 50 but responded well to treatment with prednisolone in addition to CSA. Discontinuation of prednisolone and CSA did not induce GVHD recurrence. As previously described [9], MRD analyses using real-time quantitative RT-PCR (RQ-PCR) analysis of bone marrow samples were performed monthly from the start of the conditioning regimen. The patient gave written informed consent to participate in this study to assess the utility of MRD analysis after allogeneic SCT, which was reviewed and approved by an institutional review board at Kanazawa University Medical Center. The detection threshold of RQ-PCR in this study is 50 copies/1 μg RNA. The predictive value of the BCR-ABL transcript number for hematological relapse of Philadelphia-ALL in this setting is ≥50 copies/1 μg RNA (unpublished data). The MRD study showed the patient attaining molecular remission on day 28, as defined by a decrease in BCR-ABL transcripts below the detection threshold of RQ-PCR (Figure 1). Molecular evidence of recurrent leukemia on day 95 resulted in the re-initiation of treatment with 600 mg imatinib on day 103. Despite the subsequent detection of bone marrow BCR-ABL fusion-positive cells by fluorescence in situ hybridization (FISH), the patient regained molecular remission with imatinib monotherapy on day 120. The patient discontinued imatinib on day 146 due to the development of imatinib-induced pericardial effusion. Donor leukocyte infusions (DLI) on days 168 and 196 containing 1.2 $\times$ 10^8/kg and 1.3 $\times$ 10^8/kg CD3⁺ cells, respectively, from the same donor resulted in the reappearance of molecular relapse on day 248.

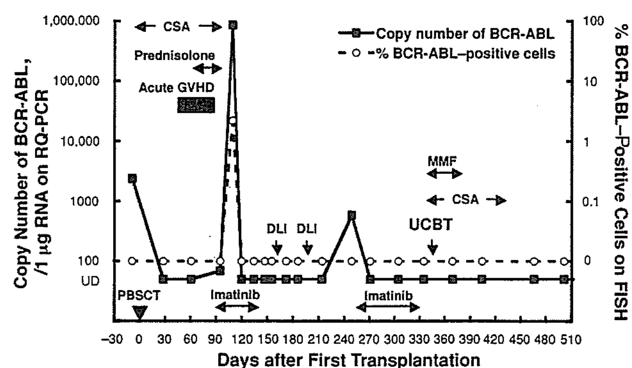

Figure 1. Results of longitudinal RQ-PCR and FISH analyses in bone marrow begun at the start of the preparative regimen for the first transplantation. The detection threshold of BCR-ABL transcripts was 50 copies/μg RNA. UD indicates undetectable levels of transcripts.

Resumption of imatinib on day 257 at a daily oral dose of 400 mg led to molecular remission again with minimal toxicity. In view of the low probability of sustained molecular remission with imatinib [4,5], the urgent need for stem cell transplantation, and the high incidence of regimen-related mortality after conventional second allogeneic SCT in patients with early relapse after first allogeneic SCT [10], reduced-intensity UCBT (RI-UCBT) was planned. The preparative regimen, based on a previous report [11], consisted of cyclophosphamide at 50 mg/kg on day –6, fludarabine at 40 mg/m^2 daily on days –6 to –2, and a single dose of 2 Gy of total body irradiation on day –1. Unrelated cord blood (UCB) that was phenotypically matched and genotypically mismatched at only the DRB1 locus was obtained through the Tohoku Cord Blood Bank. The patient received a UCB graft at a dose of 2.0 $\times$ 10^7 nucleated cells/kg of the recipient's body weight in October 2002, 343 days post–first transplantation. To prevent rejection of the graft and GVHD, CSA and mycophenolate mofetil (MMF) were started 3 days before transplantation. Granulocyte colony-stimulating factor was administered from day 1. The patient tolerated the conditioning regimen well, with neutrophil recovery (>5 $\times$ 10^8/L) occurring by day 13. Lineage-specific chimerism analysis 25 days posttransplantation showed 100% donor chimerism in both myeloid and T-lymphoid lineages. MMF was discontinued within 3 weeks of UCBT. A gradual tapering of CSA commenced on day 30, and CSA was withdrawn on day 80. On day 140, the patient developed chronic GVHD of the mouth that resolved without treatment. The patient continues to show good performance 30 months after the second transplantation and maintains molecular remission.

3. Discussion

In our patient, the early administration of imatinib, initiated upon molecular evidence of Ph⁺ ALL recurrence after allogeneic SCT, induced molecular remission that has continued after subsequent UCBT with early tapering of CSA. However, the relative contributions of MRD-oriented ima-

tinib treatment, CBT, and early tapering of CSA on her long duration of remission are uncertain.

Concerning imatinib for leukemia relapse after SCT, Wassmann et al [8] reported that in 14 (52%) of 27 Ph+ ALL patients receiving imatinib upon detection of MRD after SCT, BCR-ABL transcripts became undetectable after a median of 1.5 months. They emphasized that their 48% DFS at 18 months since MRD-triggered imatinib commencement surpassed the 5% DFS in a previous report [5] of imatinib treatment for any Ph+ ALL relapse after SCT, suggesting a superior response of imatinib in the setting of MRD. However, even with MRD-triggered imatinib after SCT, there is no plateau in survival curves [8]. These findings indicate that imatinib monotherapy is unable to maintain molecular remission in patients with Ph+ ALL, despite the benefit that treatment with imatinib may provide patients in relapse with a good platform, molecular remission, for subsequent treatment strategies such as SCT.

Takahashi et al [12] showed better outcomes in acute GVHD, treatment-related mortality, and DFS after UCBT than after BMT from unrelated donors. This report may suggest that UCBT could provide the best explanation for the good clinical course in our patient. Thus far, 2 patients with Ph+ ALL receiving cord blood grafts have been reported [13,14]. Wang et al [14] reported an 11-year-old male patient who received HLA-identical sibling donor CBT during hematologic relapse after chemotherapy. The patient relapsed on day 117 and died of leukemia on day 146. The second was a 3-year-old girl who received HLA 1-antigen-mismatched UCBT during the first hematologic CR [13]. She had maintained long-term remission, but died of leukemia 29 months after UCBT (personal communications). The present case is the first reported case of CBT used to treat a patient with a prior history of SCT. These observations are insufficient to determine the effectiveness of CBT for Ph+ ALL. However, given that no curative treatment has been established for patients with Ph+ ALL relapsing after allogeneic SCT [1-4,8], we suggest that CBT could become a promising therapeutic option for the management of such patients.

Our patient was tapered off CSA early after UCBT in an attempt to reduce the chance of relapse due to an enhanced GVL effect. This resulted in successful durable remission without the development of GVHD severe enough to require immunosuppressive therapy. Despite the induction of the GVL effect in some patients with advanced disease, the rapid tapering of CSA could place patients at a risk of developing fatal GVHD [15]. However, the immunological naivety [16] of cord blood lymphocytes may decrease the probability of intractable GVHD after UCBT, allowing the safe reduction of posttransplantation immunosuppression, while the shortened duration of immunosuppression may permit lymphocytes to exert a more potent antileukemic effect. This hypothesis is supported by clinical observations showing similar rates of disease relapse and lower rates of acute and chronic GVHD in adult patients receiving UCBT compared to those receiving allogeneic bone marrow transplantation or PBSCT [11,12,17-19]. In further support, a case report of a child with blast crisis CML was successfully treated with related cord blood transplantation and early withdrawal of CSA [20]. However, it is still unclear whether early tapering of immunosuppression therapy was instrumental in the maintenance of molecular remission in our patient. The correlation of the early tapering of immunosuppression therapy with the sustained molecular remission is only speculative.

Patients undergoing a second allogeneic SCT due to the recurrence of Ph+ ALL have a very poor prognosis because of increased regimen-related toxicity and a high rate of relapse. With the intention of avoiding severe toxicity, we used a reduced-intensity conditioning regimen that was well-tolerated and achieved durable donor engraftment with minimal GVHD in accordance with results in previous reports of RI-UCBT [11,19].

DLI has a very limited success rate in Ph+ ALL relapsing after allogeneic SCT [21], likely due in part to a leukemia burden too high at relapse to be eradicated by DLI. Accordingly, the monitoring of MRD after allogeneic SCT is useful for the maximizing antileukemic effects of DLI as well as those of a second transplant. Evidence to this effect can be seen in the reports of 2 patients with MRD levels of leukemia relapse after allogeneic SCT who obtained molecular remission following DLI [22,23]. Recently, Shimori et al [24] reported 2 patients with CML relapsing into lymphoid blast crisis and with Ph+ ALL, in which the initiation of imatinib led to the elimination of BCR-ABL fusions that was maintained after DLI. However, taking into consideration the persistence of BCR-ABL transcripts after DLI in both patients, the continuation of imatinib treatment, and the short-term follow-up within 5 months of imatinib treatment, it cannot be determined whether these effects are due to imatinib alone rather than the combination of imatinib and DLI. In conjunction with our observation that DLI was ineffective in a patient with molecular remission induced by imatinib prior to DLI, the efficacy of DLI in combination with imatinib remains unclear at present.

The advantages of UCBT are the immediate availability of cells, the absence of a risk to the donor, and a reduced need for HLA compatibility between the donor and recipient [11,2,17-19,25,26]. Because of the establishment of many cord banks, nearly every patient can find a potential cord blood graft, suggesting that a therapeutic approach using imatinib and UCBT guided by molecular monitoring for MRD after SCT could be applied in the majority of patients with Ph+ ALL. A subsequent study of a large group of patients is required to assess whether imatinib in combination with UCBT is a safe and effective therapy for patients with molecular evidence of recurrent Ph+ ALL after allogeneic SCT.

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ORIGINAL ARTICLE

Reduced-intensity unrelated cord blood transplantation for treatment of metastatic renal cell carcinoma: first evidence of cord-blood-versus-solid-tumor effect

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We report a 69-year-old man with cytokine-resistant metastatic renal cell carcinoma treated with reduced-intensity unrelated cord blood transplantation. The patient achieved durable donor engraftment with minimal graft-versus-host disease. The patient showed regression of metastatic disease, providing the first evidence of a graft-versus-tumor effect on a solid tumor resulting from cord blood graft.

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Keywords: reduced-intensity unrelated cord blood transplantation; metastatic renal cell carcinoma; graft-versus-tumor effect

Introduction

Metastatic renal cell carcinoma (RCC) is resistant to standard radiotherapy or chemotherapy, and patients with this disease have a poor outlook.[1] Although immunotherapy with cytokines such as interleukin 2 and interferon alpha can lead to regression of RCC in some patients, the response rate for these treatments remains around 10–20%, and response is usually temporary.[2] Recently, allogeneic stem cell transplantation utilizing mobilized peripheral blood from a matched donor has been investigated as an alternative immunotherapeutic strategy for the treatment of advanced RCC. The results of pilot reduced-intensity transplant trials for metastatic RCC are encouraging and show that responses can occur in patients with advanced metastatic disease that has failed to respond to conventional cytokine-based therapy.[1,3 15]

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Unrelated cord blood (UCB) is considered an alternative hematopoietic stem cell source for transplantation, and its use in adult patients with hematologic disorders is increasing.[16 21] Thus far, UCB transfer has not been attempted in patients with a solid-organ malignancy such as RCC. Here, we report a patient with metastatic RCC treated with reduced-intensity unrelated cord blood transplantation (RI-UCBT).

A 56-year-old man with clear cell RCC of his right kidney underwent a right nephrectomy in March 1991. Six years later, metastatic diseases were found in the right upper jaw and pancreas and were partially removed. The remaining metastases grew and new metastases developed in the left lung, left kidney, retroperitoneal space and subcutaneous space. The patient was treated with a 12-week course of combination therapy of subcutaneous interferon alpha 2 MU/m^2 and interferon gamma 2 MU/m^2 five times per week. However, these metastases showed a progressive increase in the size. Because of the low probability of response to further conventional treatment for metastatic RCC, the patient was referred to our institute in February 2004 at the age of 69 years. Then, serum LDH level was 286 IU/l (normal range, 0–250), hemoglobin level 10.6 g/dl, serum calcium level 9.3 mg/dl and erythrocyte sedimentation rate 38 mm/h. Reduced-intensity allogeneic stem cell transplantation was considered in order to decrease regimen-related toxicity, but because of the lack of a suitable donor candidate among his family members, unrelated RI-UCBT was planned. The patient gave written informed consent to participate in an institutional review board-approved investigational protocol designed to evaluate graft-versus-tumor (GVT) effects in metastatic RCC after nonmyeloablative allogeneic transplantation. The preparative regimen, which was based on a previous report,[22] consisted of cyclophosphamide, 50 mg/kg, on day −6, fludarabine, 40 mg/m^2, daily on days −6 to −2, and a single dose of 200 cGy of total body irradiation on day −1. UCB, phenotypically mismatched at one HLA-B antigen and one DRB1 antigen, was obtained through the Japanese Cord Blood Bank Network (J-CBBN). The patient received a UCB graft at a dose of 2.0×10^7 nucleated cells/kg of recipient body weight in

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March 2004. To prevent rejection of the graft and graft-versus-host disease (GVHD), intravenous cyclosporine A (1.5 mg/kg b.i.d.) and oral mycophenolate mofetil (15 mg/kg b.i.d. until neutrophil engraftment) were started 3 days before transplantation. Granulocyte colony-stimulating factor (G-CSF) was initiated on day 1. The patient developed poor engraftment with at most 50% of peripheral blood granulocytes of donor origin. This resulted in graft rejection, with complete autologous recovery on day 41. One hundred and six days after first transplant, the patient received a second UCB graft from J-CBBN containing 2.2×10^7 nucleated cells/kg of recipient body weight, which was phenotypically mismatched at one HLA-B antigen and one DRB1 antigen. Conditioning therapy consisted of fludarabine, 25 mg/m^2, daily on days −7 to −3, melphalan, 80 mg/m^2, on day −2 and a single dose of 400 cGy of total body irradiation on day −1, as previously reported.[18] A continuous infusion of tacrolimus, 0.03 mg/kg, was started from 3 days before transplant for prophylaxis of GVHD and graft rejection. G-CSF was started on day 1. The patient tolerated the conditioning regimen well and exhibited rapid engraftment, with neutrophil rising above 5×10^8/l by day 15. Chimerism analysis of blood on day 20 after second transplant revealed 100% donor origin in both myeloid and T-lymphoid lineages. On day 47, grade II acute GVHD of the skin and gut developed. Acute GVHD improved rapidly after increasing doses of tacrolimus without corticosteroid therapy, but it became dependent on the treatment of tacrolimus. The tacrolimus was finally tapered off at 11 months, and thereafter no GVHD developed. Treatment response was evaluated monthly after transplantation according to the Response Evaluation Criteria in Solid Tumors (RECIST).[23] A computed tomography (CT) scan at 2 months showed substantial regression of metastasis in the left kidney and retroperitoneal space (Figure 1), and the patient was determined as partial remission (PR). The PR had lasted for 3 months until new metastatic lesions in the liver and pancreas appeared at 5 months after second transplantation, defined as progressive disease (PD). At the onset of PD, he had active GVHD of the gut, which was treated with oral tacrolimus alone. Metastatic lesions progressed in size very slowly until 18 months after second transplantation, but since then, they have been unchanged until the time of this writing. The association of the onset of GVHD with the development of PR as well as that of discontinuation of the immunosuppression with no further progression of disease is suggestive of a GVT effect in this patient. The patient continues to show a good performance 26 months after second transplantation without active GVHD.

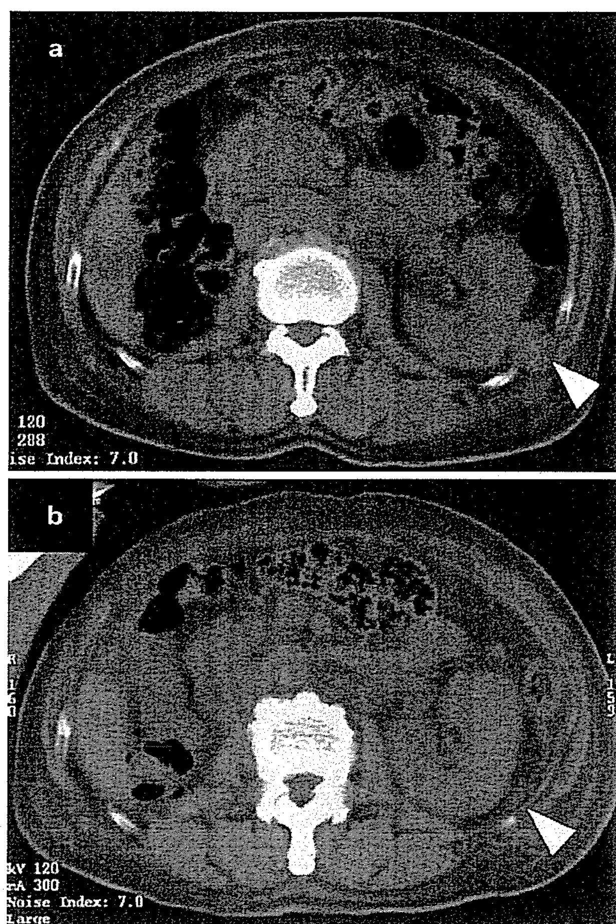

Figure 1 CT images of retroperitoneal metastasis (arrowheads) in a patient before second transplantation (a) and 2 months after second transplantation (b). Regression in the patient was concordant with the onset of acute GVHD of the skin and gut.

Discussion

Metastatic RCC is the solid tumor in which a GVT effect has been most expected. Childs *et al.*[3] and Childs and Otterud[24] have reported that of 50 patients with metastatic RCC who underwent allogeneic peripheral blood stem cell transplantation (PBSCT), 22 (44%) showed a disease response including four complete responses and 18 PRs, and five (10%) patients had a mixed response. However, the worldwide clinical experience of allogeneic SCT for metastatic RCC is limited, with approximately 200 cases reported in the literature.[3,15,24] One of the major restrictions of this approach is the requirement that transplant candidates have an HLA-identical related donor. This requirement may limit the use of allogeneic stem cell transplantation to a minority of patients with metastatic RCC. Our patient achieved long-term survival following RI-UCBT, despite the lack of a suitable donor candidate among his family members.

Cord blood, which is collected from the umbilical cord and placenta of healthy newborns, is an alternative source of hematopoietic stem cells.[25] Compared to adult peripheral blood or bone marrow, cord blood contains a greater proportion of highly proliferative hematopoietic progenitor cells,[26] which may account for myeloid and lymphoid reconstitution after cord blood transplantation (CBT) despite the presence of fewer cells (by 1–2 logs) in cord blood than in bone marrow or mobilized peripheral blood.

It was originally thought in CBT that the immunological naivety of cord blood lymphocytes[26] might produce a

lowered GVT effect at the expense of a lower GVHD incidence. However, clinical studies revealed similar rates of disease relapse and lower rates of acute and chronic GVHD in adult patients with hematologic malignancies receiving CBT compared to those receiving allogeneic bone marrow transplantation or PBSCT.[17,19,21] Although it remains unclear whether such favorable effects also occur in patients with metastatic RCC who undergo CBT, several observations support the hypothesis that similar alloimmune effects mediated by donor T cells could work in these patients.[24,27-30] Although the target antigens in GVT effects after allogeneic transplantation against metastatic RCC have not been determined, clinical and laboratory observations suggested that minor histocompatibility antigens (mHAs) could be mainly involved as target antigens in GVT effects for metastatic RCC after PBSCT, and donor T cells responding to mHAs could be generated.[24,27,28] The fact that cord blood can generate cytotoxic T cells specific for the mHA in the same way as peripheral blood and bone marrow[29,30] might imply that mHA-specific donor T cells contributive to a GVT effect against metastatic RCC are inducible in a patient receiving a cord blood graft as well.

As allogeneic stem cell transplantation is associated with many and sometimes severe toxic effects, we used a reduced-intensity conditioning regimen as described in previous reports,[18,22] which included low-dose total body irradiation in combination with cyclophosphamide and fludarabine or with melphalan and fludarabine. This RI-UCBT regimen proved to be well tolerated and achieved durable donor engraftment with minimal GVHD. Although our patient required a second RI-UCBT because of graft refection after first RI-UCBT, the demonstrated feasibility of secondary transplantation may be of benefit in the treatment of older cancer patients with RI-UCBT. Of note, the observation in the patient that retroperitoneal and renal metastasis regressed, despite a mixed response, provides the first evidence of a GVT effect by a cord blood graft on RCC.

The advantages of CBT are the immediate availability of cells, the absence of risk to the donor and a lower need for HLA compatibility between the donor and the recipient.[16-22] Because of the establishment of many cord blood banks, nearly every patient can find a potential cord blood graft, suggesting that CBT could substantially expand the use of allogeneic transplantation in patients with metastatic RCC. Despite these potential advantages, there are several disadvantages such as susceptibility to graft rejection, prolonged recovery of hematopoiesis and unavailability of donor lymphocyte infusions. A clinical study focusing on minimizing toxicities and controlling infectious complications as well as enhancing GVT effects is needed to optimize the success of CBT for treatment of advanced RCC.

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EXPERIMENTAL HEMATOLOGY

Roles of DRB1*1501 and DRB1*1502 in the pathogenesis of aplastic anemia

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Objective. **Although a number of reports have documented a significantly increased incidence of HLA-DR15 in aplastic anemia (AA), the exact role of HLA-DR15 in the immune mechanisms of AA remains unclear. We herein clarify the difference between DRB1*1501 and DRB1*1502, the two DRB1 alleles that determine the presentation of HLA-DR15, in the pathophysiology of AA.**

Materials and Methods. **We investigated the relationships of the patients* HLA-DRB1 allele with both the presence of a small population of $CD55^-CD59^-$ (PNH-type) blood cells and the response to antithymocyte globulin (ATG) plus cyclosporin (CsA) therapy in 140 Japanese AA patients.**

Results. **Of the 30 different DRB1 alleles, only DRB1*1501 (33.6% vs 12.8%, $p_c < 0.01$) and DRB1*1502 (43.6% vs 24.4%, $p_c < 0.01$) displayed significantly higher frequencies among the AA patients than among a control. AA patients possessing HLA-DR15 tended to be old, and especially, the frequency of DRB1*1502 in patients 40 years of age and older (52.4%) was markedly higher than that in those younger than 40 years old (16.2%, $p_c < 0.01$). Only DRB1*1501 was significantly associated with the presence of a small population of PNH-type cells and it also showed a good response to ATG plus CsA therapy in a univariate analysis. A multivariate analysis showed only the presence of a small population of PNH-type cells to be a significant factor associated with a good response to the immunosuppressive therapy ($p < 0.01$).**

Conclusions. **Although both DRB1*1501 and DRB1*1502 contribute to the development of AA, the methods of contribution differ between the two alleles.** **© 2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

Aplastic anemia (AA) is a syndrome characterized by pancytopenia and bone marrow hypoplasia. Although the etiology remains unclear, the immune destruction of hematopoietic stem cells has been considered the most important mechanism of bone marrow failure in AA [1]. One important finding supporting the role of such autoimmune mechanisms in AA is the high incidence of a certain HLA allele in AA patients. A number of reports have documented a significantly increased incidence of HLA-DR2 or the split antigen HLA-DR15 in AA [2–5]. We previously demonstrated a strong association between DRB1*1501 and a susceptibility to AA, in which the hematopoietic function improves with administration of cyclosporin A (CsA) [6]. Some reports have also demonstrated that HLA-DR15 or DRB1*1501 can predict the response to immunosuppressive therapy (IST) in patients with AA and myelodysplastic syndrome (MDS) [7–9], while others have failed to identify HLA-DR15 as a predictor for the response to antithymocyte globulin (ATG) therapy [3,10,11].

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In our previous study, AA patients carrying DRB1*1502, another major allele corresponding to HLA-DR15 in Japanese, did not show a better response to CsA than those without HLA-DR15 [6]. The exact role of HLA-DR15 in the immune mechanisms of AA thus remains unclear, probably because of the low number of patients that have been studied for DRB1 alleles and the general heterogeneity in the pathogenesis of AA.

Another interesting aspect of HLA-DR15 is the association with the expansion of paroxysmal nocturnal hemoglobinuria (PNH) clones. Several studies have revealed the frequency of HLA-DR15 to be significantly higher in patients with AA and MDS possessing PNH-type blood cells and in florid PNH than in normal controls [10,12], however, the relationship between DRB1 alleles corresponding to DR15 and increased PNH-type cells in AA has not yet been studied in detail. The close relationship between HLA-DR15 and the expansion of PNH clones suggests that the T-cell responses against certain antigen presented by HLA-DR15 or other HLA-class II alleles in linkage disequilibrium with DR15 in hematopoietic stem cells may cause bone marrow failure, thus allowing PNH-type stem cells to survive.

We previous demonstrated the frequency of HLA-DR15 to markedly increase in patients with MDS-refractory anemia (RA) and a small population of PNH-type cells (>0.003% for granulocyte, >0.005% for red blood cells [RBCs]), as demonstrated by sensitive flow cytometry [13]. In that study, RA patients possessing a small population of PNH-type cells displayed favorable responses to CsA. An investigation of a large number of AA patients treated with IST using the same methods to detect small populations of PNH-type cells would thus clarify the role of DRB1 alleles corresponding to HLA-DR15 and PNH-type cells in the immune mechanisms of AA and their mutual relationships. To test this hypothesis, we investigated the relationship between the DRB1 allele in such patients and both the presence of a small population of PNH-type cells and the response to ATG plus CsA therapy in 140 Japanese AA patients.

Materials and methods

Patients

Table 1 summarizes the patient characteristics. The 140 Japanese AA patients were diagnosed at Kanazawa University Hospital, hospitals that participate in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions from April 1999 through November 2005. The study subject included 77 patients who were tested for any correlation between the presence of a minor population in PNH-type cells and the response to IST in our previous study [14]. The severity of AA was classified according to the criteria proposed by Camitta [15] and Marsh et al. [16]. All participants provided written, informed consent to all procedures associated with the study, which was approved by the Ethical Committee at our institution (study number 46). This study also conforms to the recently revised tenets of the Helsinki protocol.

Table 1. Patient characteristics

Characteristics	n	Range
Total (n)	140	NA
Age at diagnosis (y)	60	12–92
Gender: Male/female	65/75	NA
Severity: Severe/moderate	65/75	NA
Neutrophil count ($\times 10^9$/L)	720	0–2226
Platelet count ($\times 10^9$/L)	20	2–118
Reticulocyte count ($\times 10^9$/L)	28	2–106
No. of patients with clonal abnormality (n)	11	NA

NA = not applicable.

Detection of PNH-type cells

We performed two-color flow cytometry of the granulocytes and RBCs according to our previously described method [14,17,18]. First, 3–5 mL heparinized blood was drawn from each patient. To detect the PNH-type granulocytes, phycoerythrin (PE)-labeled anti-CD11b monoclonal antibodies (mAbs; Becton Dickinson, Mountain View, CA, USA), fluorescein-isothiocyanate (FITC)-labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA, USA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen) were used in combination with isotype-matched control mAbs, as described previously. To detect PNH-type RBCs, PE-labeled anti-glycophorin A mAbs (clone JC159, DAKO, Glostrup, Denmark) were used instead of anti-CD11b mAbs . Fresh blood was diluted to 3% using phosphate-buffered saline, and 50 mL diluted blood was incubated with 4 mL PE-labeled anti-glycophorin A mAbs , FITC-labeled anti-CD55 and anti-CD59 mAbs on ice for 25 minutes. A total of at least 1×10^5 CD11b$^+$ granulocytes and glycophorin A$^+$ RBCs within each corresponding gate were analyzed using FACScan flow cytometry (Becton Dickinson). In order to avoid any false-positive results, we excluded CD11b^{dim} and glycophorin A^{dim} cells from the analyses using careful gating because these cells include damaged cells those are often mistakenly judged to be PNH-type cells because of their poor binding to anti-CD55 and anti-CD59 mAbs. This flow cytometry method failed to detect 0.003% or more CD55$^-$CD59$^-$CD11b$^+$ granulocytes or 0.005% or more CD55$^-$CD59$^-$glycophorin-A$^+$ RBCs in any of 183 healthy individuals. We, therefore, defined the presence of >0.003% CD55$^-$CD59$^-$CD11b$^+$ granulocytes CD55$^-$CD59$^-$glycophorin-A$^+$ RBCs to be abnormal [14,18].

Determination of DRB1 alleles

DRB1 alleles of 140 AA patients and 491 healthy Japanese randomly selected from general population [19] were determined using polymerase chain reactions with sequence-specific primers (PCR-SSP) (Micro SSP HLA DNA typing trays; One Lambda, Canoga Park, CA, USA). Genomic DNA was prepared from blood samples using a DNA extraction kit (Generation capture column kit; Gentra, Minneapolis, MN, USA).

ATG plus CsA therapy and response criteria

Seventy-seven of 140 patients (55.0%) were treated with ATG (15 mg/kg/day, 5 days; Lymphoglobuline, Aventis Behring, King of Prussia, PA, USA) and CsA (Novartis, Basel, Switzerland, 6 mg/kg/day) within 1 year of diagnosis. The dose of CsA was adjusted to maintain trough levels at between 150 and 250 ng/mL and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (filgrastim, 300 $\mu g/m^2$ or lenograstim, 5 μg/kg) was administered to some patients. The response to ATG plus CsA therapy was evaluated according to the response criteria described by Camitta [20]. A complete response was defined as hemoglobin normal for age, neutrophil count $>1.5 \times 10^9/L$, and platelet count more than $150 \times 10^9/L$. A partial response was defined as transfusion-independent and no longer meeting criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling of the normalization of at least one cell line or an increase in the baseline hemoglobin of more than 30 g/L (if initially <60 g/L), a neutrophil count of $>0.5 \times 10^9/L$ (if initially $<0.5 \times 10^9/L$), and a platelet count of more than $10 \times 10^9/L$ (if initially $<20 \times 10^9/L$) in patients with moderate AA.

Statistical analysis

The allele frequency defined as the proportion of patients with at least one copy of a specific gene was determined by direct counting. The χ^2 test compared the allele frequencies of HLA-DRB1 between the patient groups and a Japanese control population, composed of 491 healthy unrelated individuals selected at random from the general population [19]. The corrected value of p (p_c) was calculated by multiplying p with the number of alleles tested (n = 30). The χ^2 test, Fisher's exact test, and logistic procedures [21] analyzed associations between prevalence of increased PNH-type cells and genetic factors, and between individual pretreatment variables and the response to ATG plus CsA therapy. The Kaplan-Meier methods graphically compared the cumulative incidence of the response to ATG and CsA therapy and the time to event, while the log-rank test analyzed differences between the patients who possess HLA-DRB1*1501, DRB1*1502 and DRB1 alleles other than these two alleles. All statistical analyses were performed using the JMP version 5.0.1J software program (SAS Institute, Cary, NC, USA).

Results

Frequencies of DRB1 alleles in AA patients

Table 2 summarizes the frequencies for the 30 different DRB1 alleles identified in the 140 AA patients and 491 controls. Only the frequencies of DRB1*1501 (33.6% vs 12.8%, $p_c < 0.01$, odds ratio = 3.43) and DRB1*1502 (43.6% vs 24.4%, $p_c < 0.01$, odds ratio = 2.39) were significantly higher among the AA patients than among controls. Figure 1 illustrates the numbers of patients with DRB1*1501 and/or DRB1*1502 and the patients without either of the two alleles in the different age groups. Two peaks in the age distribution of the patients were noted, namely, at 20 to 29 years old and at 60 to 79 years old. After dividing the patients into young (younger than 40 years old, n = 37) and old (40 years or older, n = 103) groups, 82.5% of patients in the older group carried at least one of DRB1*1501 or DRB1*1502. Frequency of DRB1*1502 in the older group (54 of 103 patients, 52.4%) was significantly higher (p_c = 0.03) than that in the younger group (6 of 37 patients, 16.2%). No significant difference in the frequency of DRB1*1501 was identified between the two groups (36 of 103 patients, 35.0% vs 11 of 37 patients, 29.7%, p = 0.56).

Table 2. Frequencies of HLA-DRB1 alleles in Japanese AA patients and controls

	AA patients (n = 140)		Controls (n = 491)		
HLA-DRB1 allele	n	%*	n	%*	p_c value**
0101	10	7.1	64	13.0	NS
0301	0	0.0	4	0.8	NS
0401	2	1.4	17	3.5	NS
0403	4	2.9	18	3.7	NS
0404	0	0.0	2	0.4	NS
0405	35	25.0	129	26.3	NS
0406	5	3.6	32	6.5	NS
0407	2	1.4	2	0.4	NS
0409	0	0.0	1	0.2	NS
0410	1	0.7	17	3.5	NS
0701	0	0.0	2	0.4	NS
0801	0	0.0	0	0.0	NS
0802	6	4.3	36	7.3	NS
0803	8	5.7	84	17.1	NS
0901	36	25.7	148	30.1	NS
1001	2	1.4	2	0.4	NS
1101	7	5.0	22	4.5	NS
1201	7	5.0	34	6.9	NS
1202	2	1.4	12	2.4	NS
1301	0	0.0	4	0.8	NS
1302	11	7.9	61	12.4	NS
1401	2	1.4	21	4.3	NS
1402	0	0.0	2	0.4	NS
1403	4	2.9	13	2.6	NS
1405	4	2.9	18	3.7	NS
1406	2	1.4	10	2.0	NS
1407	0	0.0	1	0.2	NS
1501	47	33.6	63	12.8	<0.01
1502	61	43.6	120	24.4	<0.01
1602	2	1.4	4	0.8	NS

AA = aplastic anemia; NS = not significant.

*Allele frequencies were determined by dividing the number of patients carrying one or two specific alleles by the total number of individuals.

**Corrected p value (p_c) was calculated by multiplying the p value with the number of alleles (n = 30) tested.

Prevalence of patients possessing PNH-type cells

A wide range of PNH-type granulocytes (0.005–23.0%; median, 0.153%) and PNH-type RBCs (0.007–6.57%; median, 0.094%) were detected in 92 of 140 (65.7%) AA patients. When patients were divided into four groups according to presence of DRB1*1501 and DRB1*1502, the proportions of PNH^+ patients were 66.7% (4 of 6 patients) in the