Patient No. 8, who had been treated with foscarnet for CMV antigenemia, required hemodialysis and discontinuation of CDV treatment. Patient No. 11 had grade 2 renal toxicity and CDV treatment was terminated. In contrast, patient No. 5 had grade 2 renal toxicity, but could continue CDV treatment. Patient No. 10 developed veno-occlusive disease (VOD) during CDV treatment.

Virally associated findings during the treatment with CDV

Table 1 showed donor/recipient CMV and recipient HSV serostatus. As CDV has been reported to have significant anti-CMV and anti-HSV activity, concurrent use of acyclovir (ACV) or GCV was avoided to reduce renal toxicity. Patient No. 5 developed CMV antigenemia when CDV treatment was started, and CMV antigenemia persisted during CDV treatment. After completion of CDV treatment, he was treated with GCV, which abolished CMV antigenemia. Before CDV treatment, patient No. 6 was treated with GCV for CMV antigenemia that persisted throughout CDV therapy. Patient No. 12 developed CMV antigenemia during CDV treatment. After completion of CDV treatment, CMV antigenemia was abolished by treatment with foscarnet. Patient No. 16 developed CMV antigenemia during CDV treatment, and because of an increase in CMV antigenemia GCV was added. During CDV treatment, patients No. 5 and No. 12 developed HSV-1 stomatitis, which was treated successfully with ACV (Table 3).

Discussion

The present study reports the outcome in AdV HC treated with CDV. As expected, the main toxicity of CDV treatment was renal. Among 14 evaluable patients, two developed severe renal toxicity, resulting in discontinuation of CDV treatment. One of these patients who required hemodialysis had a history of foscarnet treatment. Previous treatment with foscarnet has been reported to exacerbate CDV renal toxicity,[23] which was proved for this patient. CDV renal toxicity complicating treatment of AdV HC is difficult to evaluate. Many other nephrotoxic agents, including cyclosporine, tacrolimus, and amphotericin B, are frequently administered to HSCT patients; furthermore, AdV infection itself can cause renal damage such as nephritis[16] and obstructive nephropathy.[24] In this study, six patients (Nos. 5, 9, 12, 14, 15, and 16) experienced increased level of serum creatinine concentrations, but continued CDV treatment, with improvement in terms of both AdV HC and renal function (Table 3). Thus, AdV HC itself may have contributed to the increase in serum creatinine during CDV treatment. Use of CDV before emergence of renal damage from AdV infection would be desirable. Patient No. 10 developed VOD, which has not been reported previously as a form of CDV toxicity. More information is necessary to determine whether or not VOD is among CDV toxicities.

Among 14 evaluable patients, 10 (71%) showed clinical improvement of AdV HC, which is similar to a success rate of 63% reported in patients with definite AdV disease reported by the European Group for Blood and Marrow Transplantation.[25] A long delay between AdV infection and treatment has been linked to a greater risk of treatment failure.[17] For rapid diagnosis, we used immunochromatography. At the onset of HC, all patients in the study were positive for AdV by this method. Positivity was confirmed later both by PCR result and by isolation of AdV from urine. Thus, immunochromatography appears reliable for rapid diagnosis of AdV HC. Since post-transplant AdV infection causes significant mortality[6,9–11] and HC causes considerable patient discomfort, CDV would appear to be beneficial treatment while maintaining an acceptable toxicity profile.

At a dose of 5 mg/kg/week, CDV has been reported to have significant anti-CMV and anti-HSV activity.[26] Indeed, CDV is considered a second-line treatment for GCV-refractory CMV disease.[27] Among our patients, two had persistent CMV antigenemia and two developed CMV antigenemia during treatment with CDV. In addition, two patients developed HSV-1 stomatitis. Thus, CDV at a dose of 1 mg/kg/day three times weekly may be insufficient to prevent or treat CMV or HSV disease. Alternatively, patients who develop AdV HC might be immune compromised to the extent that for them CDV treatment may not be effective against CMV or HSV. Vigilance against infection by and/or additional prophylaxis agents for herpesviruses, therefore, is important during CDV treatment with 1 mg/kg three times weekly.

In conclusion, CDV at a dose of 1 mg/kg/day, three times weekly could be administered with acceptable toxicity for effective treatment of AdV HC. Prospective randomized trials are necessary to further study the use of CDV for AdV HC.

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International Journal of
HEMATOLOGY

Reconstitution of HLA-A*2402–Restricted Cytomegalovirus-Specific T-Cells following Stem Cell Transplantation

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Received July 26, 2004; received in revised form September 7, 2004; accepted September 10, 2004

Abstract

Cytomegalovirus (CMV)-specific immune reconstitution early after stem cell transplantation (SCT) was evaluated prospectively by detecting CD8+ T-cells, which recognize the peptide QYDPVAALF in the context of HLA-A*2402. Fifteen allogeneic SCT recipients were included in the study. All recipients and donors were seropositive for CMV and had the HLA-A*2402 allele. CMV-specific T-cells were detected as early as 1 month after transplantation, and their numbers increased to peak levels 2 to 5 months after transplantation. The numbers of CMV-specific T-cells in patients who developed grade II to IV acute graft-versus-host disease (GVHD) and received corticosteroids for acute GVHD were low in the early period after allogeneic SCT. There was a trend toward earlier reconstitution of CMV-specific CD8+ T-cells in allogeneic peripheral blood SCT (PBSCT) patients than in allogeneic bone marrow transplantation patients. The contribution of T-cells in the graft to the recovery of CMV-specific immune responses was also suggested by the finding that the reconstitution of CMV-specific CD8+ T-cells was delayed in CD34-selected autologous PBSCT compared with unpurged autologous PBSCT. The reconstitution of CMV-specific CD8+ T-cells was delayed in patients with CMV disease or recurrent CMV reactivation. These observations suggest that the detection of CMV-specific T-cells with an HLA-peptide tetramer is useful to assess immune reconstitution against CMV and to identify patients at risk for CMV disease or recurrent CMV reactivation after SCT.

Int J Hematol. 2004;80:441-448. doi: 10.1532/IJH97.04109

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Key words: HLA-A*2402; Cytomegalovirus; CMV-specific T-cells; Stem cell transplantation; Tetramer

1. Introduction

Cytomegalovirus (CMV) disease is a significant cause of morbidity and mortality after stem cell transplantation (SCT) [1]. Preemptive or prophylactic therapy with ganciclovir reduces the incidence and severity of CMV disease after transplantation [1,2]. Preemptive therapy calls for ganciclovir to be given only to patients who are at high risk for CMV disease based on the detection of CMV. However, a higher incidence of CMV disease has been demonstrated with this approach than with the prophylactic ganciclovir regimen [2]. On the other hand, prophylactic ganciclovir therapy begun at engraftment has resulted in effective prevention of CMV disease in the first 100 days after transplantation, but it has also been associated with invasive fungal infections and late CMV disease [2]. A delay in the recovery of CMV-specific T-cell responses as a result of ganciclovir prophylaxis has been suggested to contribute to the occurrence of late CMV disease [3].

CMV disease remains a major concern in SCT recipients, especially when it does not respond to ganciclovir

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therapy. Ganciclovir-resistant CMV is rarely isolated from SCT recipients, compared with acquired immunodeficiency syndrome patients [4-6]. CMV disease refractory to ganciclovir therapy is considered predominantly due to the profound immunodeficiency inherent in SCT recipients [6-8].

Major histocompatibility complex (MHC)-restricted and CMV-specific cytotoxic T-lymphocytes play an important role in protection against CMV disease [1,9-11]. Adoptive transfer of CMV-specific T-cells provides persistent reconstitution of CMV-specific T-cell responses and is effective in the treatment of patients who experience persistent or recurrent CMV infection [9-11]. A functional assay using the enzyme-linked immunospot method and flow cytometric analysis was developed to detect CMV-specific T-cells producing intracellular cytokine [12-15]. Recently, direct visualization of CMV-specific $CD8^+$ T-cells was introduced with the development of fluorescently labeled tetrameric MHC-peptide complexes [16,17]. This assay is highly sensitive and rapid for monitoring CMV-specific T-cells [18]. The HLA-A*0201–restricted NLVPMVATV epitope and the HLA-B*0702–restricted TPRVTGGGAM epitope have been used to monitor CMV-specific T-cells following allogeneic SCT [16,17]. A CMV-specific cytotoxic T-lymphocyte epitope, QYDPVAALF, was recently identified in the amino acid sequence of the 65 kd phosphoprotein (pp65) presented by the HLA-A*2402 molecule [14]. HLA-A24 is one of the most common alleles among the Japanese, and more than 95% of the alleles among the Japanese are A*2402 [19-21]. In this study, we prospectively monitored CMV-specific $CD8^+$ T-cells that recognize the short peptide, QYDPVAALF, presented by HLA-A*2402 and evaluated CMV-specific T-cell reconstitution in HLA-A*2402 recipients who received SCT.

2. Materials and Methods

2.1. Patients

Between September 2001 and December 2002, 15 consecutive patients who were HLA-A*2402 positive and had received allogeneic SCT for hematologic malignancy were enrolled in the study. All recipients and donors were seropositive for CMV before transplantation and were positive for the HLA-A*2402 allele. The characteristics of the allogeneic SCT patients are shown in Table 1.

Four patients who received unpurged autologous peripheral blood SCT (PBSCT) and 3 patients who received CD34-selected autologous PBSCT also were studied. Two of the 4 patients who received unpurged autologous PBSCT had acute nonlymphoblastic leukemia, and the remaining 2 patients had non-Hodgkin's lymphoma. Two of the 3 patients who received CD34-selected autologous PBSCT had systemic sclerosis with interstitial pneumonia, and 1 patient had dermatomyositis with interstitial pneumonia. All patients achieved sustained engraftment and survived for more than 100 days after transplantation. Informed consent was obtained from the patients or responsible family members.

Table 1.
Characteristics of Allogeneic Stem Cell Transplantation Patients*

Male/female sex, n	8/7
Median age (range), y	36 (22-68)
Underlying disease, n	
Acute nonlymphoblastic leukemia	5
Acute lymphoblastic leukemia	2
Chronic myelogenous leukemia	2
Myelodysplastic syndrome	3
Non-Hodgkin's lymphoma	2
Natural killer cell lymphoma	1
Donor and HLA disparity, n	
Related/identical	5
Related/nonidentical†	3
Unrelated/identical‡	6
Unrelated/nonidentical§	1
Stem cell source, n	
Bone marrow	8
Peripheral blood	7
Pretransplantation conditioning, n	
Myeloablative	10
Nonmyeloablative	5
GVHD prophylaxis, n	
Cyclosporine/methotrexate	6
Tacrolimus/methotrexate	9
Grades of acute GVHD, n	
0	3
I	3
II	7
III, IV	2

*GVHD indicates graft-versus-host disease.
†One patient with a donor mismatched for the HLA-B antigen, 1 patient mismatched for HLA-A and HLA-B antigens, and 1 patient with a haploidentical donor.
‡HLA-A–, HLA-B–, and HLA-DRB1–identical unrelated donors.
§An HLA-B antigen–mismatched unrelated donor.

2.2. Stem Cell Transplantation

The stem cell sources, the regimens for pretransplantation conditioning, and prophylaxis for graft-versus-host disease (GVHD) in allogeneic SCT are shown in Table 1. Eight patients underwent bone marrow transplantation (BMT), and 7 patients had PBSCT. The myeloablative conditioning regimen consisted of 12 Gy total body irradiation and 120 mg/kg cyclophosphamide (CY) in 4 patients and 12 Gy total body irradiation, 120 mg/kg CY, and 8 g/m^2 cytosine arabinoside (Ara-C) in 2 patients. Four patients were administered 16 mg/kg busulfan and 120 mg/kg CY. Nonmyeloablative conditioning regimens included 180 mg/m^2 fludarabine and 8 mg/kg busulfan in 2 patients and 125 mg/m^2 fludarabine and 60 mg/kg CY in 3 patients. To prevent GVHD, we administered 3 mg/kg cyclosporine per day as a continuous intravenous infusion in 6 patients in combination with 10 mg/m^2 methotrexate on day 1 and 7 mg/m^2 on days 3 and 6, and we administered 0.03 mg/kg tacrolimus per day by continuous intravenous infusion to another 9 patients in combination with the same methotrexate regimen. The diagnosis and grading of acute GVHD was based on clinical criteria with histologic confirmation obtained as required [22]. Complete chimerism was confirmed 1 month after transplantation in all allogeneic SCT patients.

Autologous PBSCT for acute nonlymphoblastic leukemia was performed according to the protocol described previously, with minor modifications [23]. PBSC were collected during the hematopoietic recovery period after consolidation chemotherapy and cryopreserved without ex vivo purging until transplantation. The pretransplantation conditioning regimen consisted of 16 mg/kg busulfan, 40 mg/kg etoposide (VP-16), 700 mg/m^2 Ara-C, and 12 g/m^2 Ara-C. Granulocyte colony-stimulating factor (G-CSF) (filgrastim) was combined with this regimen. For non-Hodgkin's lymphoma patients, 500 mg/m^2 VP-16 was administered for 3 days after 3 courses of induction chemotherapy with a CHOP regimen (CY, hydroxydaunomycin, vincristine [Oncovin], and prednisone). PBSC were collected during the hematopoietic recovery with G-CSF. Then, 3 more courses of induction chemotherapy were performed with the CHOP regimen. The pretransplantation conditioning regimen consisted of 400 mg/m^2 ranimustine, 1200 mg/m^2 carboplatin, 1500 mg/m^2 VP-16, and 100 mg/kg CY. For autologous CD34-selected PBSCT, mobilization of PBSC was performed with 2 g/m^2 CY for 2 days followed by G-CSF administration. CD34$^+$ cells were selected with the CliniMACS device (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cryopreserved until transplantation. The pretransplantation conditioning regimen for CD34-selected autologous PBSCT consisted of 200 mg/kg CY.

Each patient was isolated in a room with laminar air flow, and a standard decontamination procedure was followed. Prophylaxis for bacterial, fungal, and *Pneumocystis carinii* infections consisted of fluconazole, ciprofloxacin, and sulfamethoxazole/trimethoprim. All patients were given 1000 mg/day acyclovir orally from day –7 to day 35 for the prevention of herpes simplex virus infection. All blood products from random donors were irradiated and filtered.

2.3. Monitoring of CMV Reactivation

CMV reactivation was monitored at least once a week after engraftment with the antigenemia assay and quantitative real-time polymerase chain reaction (PCR) analysis. The CMV antigenemia assay was performed according to the method described previously [24]. The degree of antigenemia was expressed as the number of CMV antigen–positive cells per 5 × 10^4 leukocytes. The extraction and amplification of CMV DNA were performed according to the method described by Machida et al [25]. Viral DNA was extracted from plasma with a QIAamp Blood minikit (Qiagen, Valencia, CA, USA) and then subjected to the PCR. The PCR was performed with a TaqMan Universal PCR master mix (PE Biosystems, Tokyo, Japan).

2.4. Diagnosis of CMV Reactivation and Disease

CMV reactivation was defined as the presence of 1 or more antigen-positive cells or ≥2 × 10^2 CMV DNA copies/mL. For the diagnosis of CMV disease, such as pneumonia, colitis, or hepatitis, positive CMV-reactivation results had to be accompanied by clinical symptoms, signs, and histologic confirmation [26].

2.5. Preemptive Therapy with Ganciclovir for the Prevention of CMV Disease

The decision to use preemptive therapy was based on positive results in the antigenemia test (≥1 antigen-positive cells per 50,000 white blood cells); intravenous infusion of ganciclovir at a dose of 10 mg/kg per day was started and continued for as long as the antigenemia persisted. G-CSF was administered when the absolute neutrophil count was <500/μL.

2.6. Tetramer Staining

MHC-peptide tetramers were produced as described previously [14]. We used a CMV-specific T-cell epitope, QYDPVAALF, in the amino acid sequence of pp65 presented by HLA-A*2402 molecules. Peripheral blood mononuclear cells (2 × 10^6), which were drawn monthly after transplantation, were stained at 37°C for 15 minutes with Tricolor anti-CD8 monoclonal antibody (Caltag Laboratories, Burlingame, CA, USA) and a tetramer concentration of 0.1 mg/mL. The stained cells were washed twice and fixed with 0.5% paraformaldehyde before flow cytometric analysis. The peripheral blood lymphocyte count was used to determine the absolute number of CMV-specific CD8$^+$ T-cells.

2.7. Statistical Analysis

The percentages and absolute numbers of CD8$^+$ T-cells binding the HLA-A*2402 QYDPVAALF tetramer in the peripheral blood were compared by means of the Spearman rank correlation test. The absolute numbers of CMV-specific CD8$^+$ T-cells after transplantation were evaluated with the Mann-Whitney *U* test. A *P* value <.05 was considered statistically significant.

3. Results

*3.1. Detection of CD8$^+$ T-Cells Binding the HLA-A*2402 QYDPVAALF Tetramer in the Peripheral Blood after Allogeneic SCT*

CMV-specific T-lymphocytes, which recognize the peptide QYDPVAALF in the context of HLA-A*2402 molecules, were monitored monthly for a year after transplantation in the first 7 patients and for 3 months after transplantation in the next 8 patients (Figure 1 and Table 2). The median percentage of HLA-peptide tetramer–positive cells in the present study was 0.08% (range, 0%-2.85%) of CD8$^+$ T-cells, in contrast to approximately 0.1% in normal CMV-seropositive individuals [14,27]. The percentages and absolute numbers of CMV-specific CD8$^+$ T-cells in the peripheral blood were strongly correlated ($r = 0.92$; $P < .0001$ by the Spearman rank correlation test [n = 108]). Therefore, we express the results as the absolute number in the following sections. The median number of CMV-specific CD8$^+$ T-cells in the first 7 patients was 0.08 × 10^6/L (range, 0-16.19 × 10^6/L) at 1 month posttransplantation. The number then increased to 0.25 × 10^6/L (range, 0-41.53 × 10^6/L) at 2 months, 0.18 × 10^6/L (range, 0.01-13.61 × 10^6/L) at 3 months, 0.48 × 10^6/L (range, 0-8.45 ×

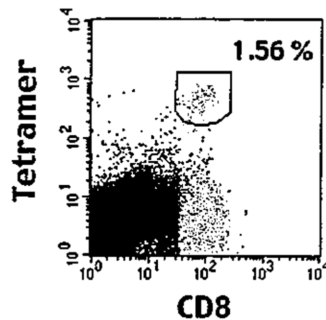

Figure 1. Staining of cytomegalovirus-specific CD8+ T-cells with an HLA-A*2402 QYDPAALF tetramer. The percentage of CD8+ T-cells binding an HLA-peptide tetramer was 1.56% of the CD8+ population.

10^6/L) at 4 months, and 0.3×10^6/L (range, 0-5.18 × 10^6/L) at 5 months posttransplantation and gradually decreased thereafter (Table 2). There was a trend toward higher numbers of CMV-specific CD8+ T-cells at 2 months after transplantation than at 1 month after transplantation among the 15 patients ($P = .08$ by the Mann-Whitney U test; Figure 2). Two of the 15 patients (unique patient numbers [UPN] 4 and 5) did not develop CMV reactivation; however, the numbers of CMV-specific T-cells in these 2 patients were increased at 2 or 3 months after transplantation (from 0.01×10^6/L at 1 month to 0.18×10^6/L at 3 months posttransplantation [UPN 4] and from 0.16×10^6/L at 1 month to 0.25×10^6/L at 2 months posttransplantation [UPN 5]) (Table 2). Thus, the increment in CMV-specific T-cells was not necessarily accompanied by detectable CMV reactivation. CMV-specific CD8+ T-cells from 2 patients were sorted by fluorescence-activated cell sorting, and PCR analysis of short tandem repeats revealed that all CMV-specific CD8+ T-cells were derived from the donor (data not shown).

3.2. Reconstitution of CMV-Specific CD8+ T-Cells in Recipients Who Developed Acute GVHD

Grades I, II, and III to IV acute GVHD developed in 3, 7, and 2 patients, respectively (Table 1). The reconstitution of CMV-specific CD8+ T-cells was significantly delayed in the patients who developed grade II to IV acute GVHD after transplantation ($P < .05$ at 1 month, $P < .01$ at 2 months, and $P < .05$ at 3 months; Mann-Whitney U test) (Figure 3). Moreover, the delayed recovery of CMV-specific CD8+ T-cells was most prominent in the patients who received ≥2 mg/kg corticosteroids for the treatment of acute GVHD ($P < .01$ at 1 month, $P < .01$ at 2 months, and $P < .01$ at 3 months posttransplantation; Mann-Whitney U test) (Figure 3). There was no difference in recovery between the patients with myeloablative conditioning regimens and those with nonmyeloablative regimens (data not shown).

3.3. Relationship between the Number of CMV-Specific CD8+ T-Cells and Stem Cell Source

Eight patients received allogeneic BMT, and 7 patients had allogeneic PBSCT. There was a trend toward an earlier reconstitution of CMV-specific CD8+ T-cells in PBSCT patients than in BMT patients ($P = .07$, Mann-Whitney U test) (Figure 4). To determine the influence of T-cells infused at transplantation on the reconstitution of CMV-specific T-cell responses, we monitored CMV-specific CD8+ T-cells in 4 CMV-seropositive patients who received unpurged autologous PBSCT and in 3 CMV-seropositive patients who received autologous PBSCT with selected CD34+ cells (Table 3). The median number of infused CD3+ cells was 11.0×10^7/kg (range, 8.8-12.8 × 10^7/kg) in the recipients who received unpurged autologous PBSCT and 3.3×10^3/kg (range, 3.0-5.0 × 10^3/kg) in the patients who received CD34-selected autologous PBSCT. The reconstitution of CMV-specific CD8+ T-cells in the patients who underwent CD34-selected autologous transplantation was delayed compared with those who underwent unpurged autologous PBSCT (Figure 4).

3.4. Reconstitution of CMV-Specific CD8+ T-Cells in Recipients Who Developed CMV Disease or Recurrent CMV Reactivation

One patient who received a BMT from an HLA-identical unrelated donor developed CMV colitis on day 26 posttransplantation. The recurrence of CMV reactivation requiring preemptive ganciclovir therapy was observed before day 100 in 3 patients. The recovery of CMV-specific CD8+ T-cells was delayed in patients with CMV disease or recurrent CMV reactivation ($P < .05$, Mann-Whitney U test) (Figure 5).

4. Discussion

The reconstitution of CMV-specific T-cell responses has previously been studied with the HLA-A*0201 and HLA-B*0702 tetramers [16-18]. In the present study, the HLA-A*2402 QYDPVAALF tetramer was used to detect CMV-specific CD8+ T-cells. HLA-A24 is one of the most common alleles among the Japanese [19-21]. CMV-specific CD8+ T-cells were detected as early as 1 month after transplantation in the peripheral blood of approximately half of allogeneic SCT recipients, including unrelated BMT recipients. Cwynarski et al [16] reported that the recovery of CMV-specific T-cells after allogeneic SCT from unrelated donors was delayed, and tetramer-binding cells were not detectable before day 100 after transplantation. In these investigators' studies, recipients of unrelated bone marrow were treated in vivo with Campath-1H as part of GVHD prophylaxis. Campath-1H induces marked lymphocytopenia after treatment. Moreover, these patients also were given ganciclovir prophylaxis, which has been reported to be correlated with failure to recover CMV-specific T-cell responses in the first 90 days after transplantation. Ganciclovir suppression of CMV replication to preclude in vivo priming and expansion of CMV-specific T-cell precursors and the inhibition of antigen-induced T-cell proliferation due to ganciclovir's effects on cellular DNA synthesis have been speculated as possible mechanisms [3]. Neither immunosuppressive drugs such as Campath-1H and antithymocyte globulin nor prophylactic ganciclovir was administered in our study. These

Table 2.
Kinetics of HLA-A*2402–Restricted Cytomegalovirus (CMV)-Specific T-Cells following Allogeneic Stem Cell Transplantation*

								CMV-Specific CD8+ T-Cells after Transplantation, ×10⁶/L											
								Months after Transplantation											
UPN	Age/Sex	Disease	Stem Cells	GVHD	Steroids†	CMV Disease	CMV Reactivation‡	1	2	3	4	5	6	7	8	9	10	11	12
1	68/M	ANLL	PBSC	0	(−)	(−)	1	16.19	41.53	13.61	8.45	2.45	3.21	2.00	2.77	1.55	2.20	3.27	1.51
2	50/M	CML	BM (UR)	II	(+)	Colitis	1	0	0.02	0.05	0.48	0.37	0.19	0.04	0	0.05	0.01	0.04	0.02
3	38/M	NHL	PBSC	0	(−)	(−)	1	0.08	15.22	8.04	6.74	5.18	16.15	11.69	5.27	6.60	5.90	2.48	2.92
4	25/F	CML	PBSC	II	(−)	(−)	0	0.01	0.05	0.18	0.06	0.09	0	0.05	0	0	0	0.08	0
5	63/F	MDS	PBSC	0	(−)	(−)	0	0.16	0.25	0.17	0.16	0.30	0	0.09	0.06	0.16	0.23	0.03	0.06
6	32/F	ALL	PBSC	I	(−)	(−)	1	1.07	1.34	0.51	0.74	0.26	0.14	0.14	0.47	0.45	0.37	0.82	1.23
7	32/M	ANLL	BM (UR)	IV	(+)	(−)	2	0	0	0.01	0	0	0	0	0	0	0.03	0.07	0.07
8	22/F	ANLL	BM (UR)	II	(−)	(−)	1	2.20	0.77	0.31									
9	37/F	ALL	BM (UR)	I	(−)	(−)	1	0	0.08	0.04									
10	36/M	MDS	BM (UR)	II	(+)	(−)	2	0	0.08	0.01									
11	32/M	NK-Ly	BM	III	(+)	(−)	2	0	0.04	0.04									
12	28/M	ANLL	PBSC	II	(+)	(−)	1	0	0.03	0.01									
13	27/F	MDS	BM (UR)	I	(−)	(−)	1	0.07	2.09	0.71									
14	49/F	NHL	PBSC	II	(−)	(−)	1	0.06	0.15	0.06									
15	48/M	ANLL	BM (UR)	II	(−)	(−)	1	0.01	0.03	0.02									

*UPN indicates unique patient number; GVHD, graft-versus-host disease; M, male; ANLL, acute nonlymphoblastic leukemia; PBSC, peripheral blood stem cells; CML, chronic myelogenous leukemia; BM, bone marrow; UR, unrelated; NHL, non-Hodgkin's lymphoma; F, female; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; NK-Ly, natural killer cell lymphoma.

†Corticosteroids ≥2 mg/kg.

‡No. of times CMV reactivation required preemptive ganciclovir therapy.

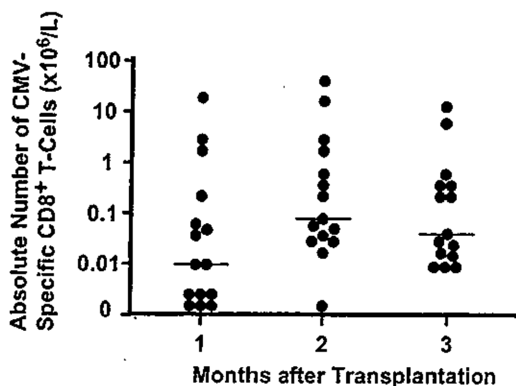

Figure 2. Reconstitution of cytomegalovirus (CMV)-specific CD8+ T-cells following allogeneic stem cell transplantation. Horizontal bars indicate median values.

factors may explain why our study detected HLA-A*2402–restricted CMV-specific CD8+ T-cells in the early period after transplantation. CMV reactivation and CMV disease frequently develop before day 100, although late-onset CMV disease is also a concern after allogeneic SCT [2,28]. Monitoring CMV-specific CD8+ T-cells in the early period after transplantation may be important to assess the immunologic recovery against CMV.

CMV reactivation has been demonstrated to precede an increase in the number of CMV-specific CD8+ T-cells, indicating a significant predictor of CMV-specific T-cell responses [16,17]. The presence of CMV in SCT recipients is also necessary for the posttransplantation reconstitution of CMV-specific T-cells [16,17]. Two of 15 patients in the present study did not develop CMV reactivation, but the recipients mounted measurable cellular immune responses against CMV at 2 months and 3 months after transplantation. The levels of CMV reactivation in the present study may also have been below the detection limits of the antigenemia assay and the quantitative real-time PCR assay, as has been suggested by Cwynarski et al [16]. Otherwise, an increase in the number of CMV-specific CD8+ T-cells may have been a result of a normal homeostatic mechanism expanding the entire T-cell pool.

The reconstitution of CMV-specific CD8+ T-cells was delayed in recipients who developed grade II to IV acute GVHD, compared with those with grade I acute GVHD or without acute GVHD. Corticosteroid administration for the treatment of acute GVHD also affected the recovery of CMV-specific CD8+ T-cells. GVHD and corticosteroids are well-recognized risk factors for CMV disease after allogeneic SCT [1,29]. The results of this study support these findings [16,17]. Careful management to prevent CMV disease is important for recipients with acute GVHD and for those who receive corticosteroids for the treatment of acute GVHD after allogeneic SCT.

CMV-specific CD8+ T-cells have been demonstrated to be derived from the donor [30]. Recurrent CMV antigenemia or CMV disease occurs more frequently after transplantation from a CMV-seronegative donor than from a seropositive donor [3,17]. Gratama et al [17] also showed that the number of CMV-specific T-cells infused into CMV-seropositive SCT recipients was inversely correlated with the number of recurrent CMV infections after transplantation. These results indicate that the number of CMV-specific CD8+ T-cells in the grafts may exert an influence on the recovery of CMV-specific T-cell responses after transplantation. In the present study, there was a trend toward an earlier reconstitution of CMV-specific CD8+ T-cells in recipients of PBSC than in bone marrow recipients. PBSC contain 5 to 10 times more T-cells than bone marrow, possibly contributing to an earlier reconstitution of CMV-specific T-cell responses in PBSCT recipients, although the exact number of T-cells infused at transplantation was not determined in this study. Hakki et al [29] demonstrated in a multivariate analysis that using bone marrow as a source of stem cells was associated with impaired CD8+ T-cell function. Moreover, the delayed recon-

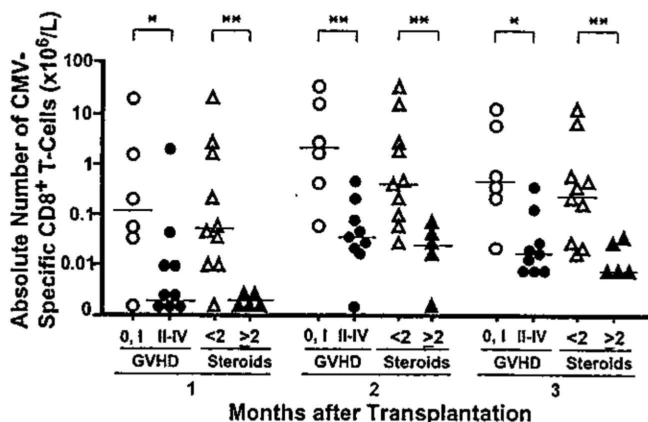

Figure 3. Reconstitution of cytomegalovirus (CMV)-specific CD8+ T-cells in patients who developed acute graft-versus-host disease (GVHD) and in those who received corticosteroids following allogeneic stem cell transplantation. Indicated are patients who received <2 mg/kg (<2) and ≥2 mg/kg (≥2) corticosteroids. Horizontal bars indicate median values (*, $P < .05$; **, $P < .01$).

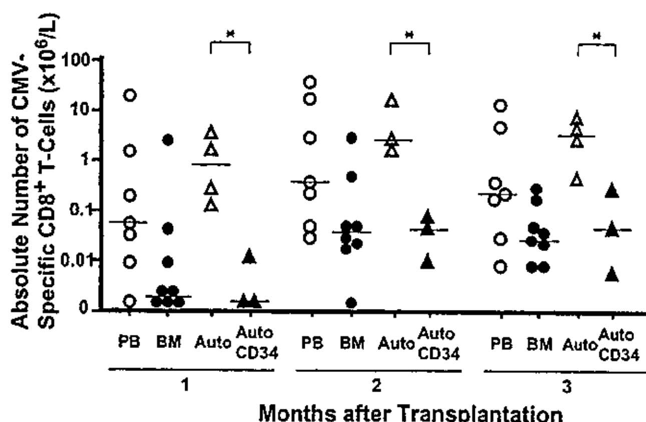

Figure 4. Reconstitution of cytomegalovirus (CMV)-specific CD8+ T-cells in patients who received peripheral blood (PB) or bone marrow (BM) and in patients who received an unpurged autologous PB stem cell transplant (PBSCT) (Auto) or CD34-selected autologous PBSCT (Auto CD34). Horizontal bars indicate median values (*, $P < .05$).

Table 3.
Characteristics of Autologous Peripheral Blood Stem Cell (PBSC) Transplantation Patients*

UPN	Age, y/Sex	Disease/Status	Stem Cells	CD34+ Cell Selection†	Pretransplantation Conditioning§	CD34+ Cells Infused, /kg	CD3+ Cells Infused, /kg
1	57/Male	NHL/1Rel	PBSC	(-)	MCEC	1.9×10^6	NE
2	52/Male	ANLL/1CR	PBSC	(-)	G-BEA	8.1×10^6	11.0×10^7
3	44/Female	NHL/1CR	PBSC	(-)	MCEC	24.7×10^6	8.8×10^7
4	30/Female	ANLL/1CR	PBSC	(-)	G-BEA	6.0×10^6	12.8×10^7
5	53/Female	SLE, SSc, IP	PBSC	(+)	CY	21.0×10^6	3.3×10^3
6	54/Female	DM, IP	PBSC	(+)	CY	4.9×10^6	5.0×10^3
7	55/Male	SSc, IP	PBSC	(+)	CY	6.6×10^6	3.0×10^3

*UPN indicates unique patient number; NHL, non-Hodgkin's lymphoma; 1Rel, first relapse; MCEC, regimen of 400 mg/m² ranimustine, 1200 mg/m² carboplatin, 1500 mg/m² etoposide, and 100 mg/kg cyclophosphamide; NE, not examined; ANLL, acute nonlymphoblastic leukemia; 1CR, first complete remission; G-BEA, regimen of 16 mg/kg busulfan, 40 mg/kg etoposide, and 700 mg/m² and 12 g/m² cytosine arabinoside, combined with granulocyte colony-stimulating factor; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; IP, interstitial pneumonia; CY, 200 mg/kg cyclophosphamide; DM, dermatomyositis.

†CD34+ cells were selected with the CliniMACS device.

stitution in the present study of CMV-specific CD8+ T-cells in the CD34-selected autologous PBSCT recipients compared with the unpurged autologous PBSCT recipients suggests that CMV-specific T-cells in the grafts contribute to the recovery of CMV-specific T-cell responses in the early period after SCT. Further study, such as an examination of the levels of the T-cell receptor excision circle, may determine whether T-cells in the graft repopulate the thymus dependently or independently in the early period after SCT [31].

The reconstitution of CMV-specific CD8+ T-cells was delayed in patients who developed CMV disease or recurrent CMV reactivation, suggesting that CMV-specific CD8+ T-cells confer protection against CMV disease or recurrent high-level CMV reactivation, although CMV-specific CD8+ T-cells have been reported to be heterogeneous in healthy seropositive donors, with only a portion of these cells correlating to functional virus-specific cells [32]. The HLA-peptide tetramer assay is useful to identify patients at risk for CMV disease or recurrent CMV reactivation requiring preemptive ganciclovir therapy.

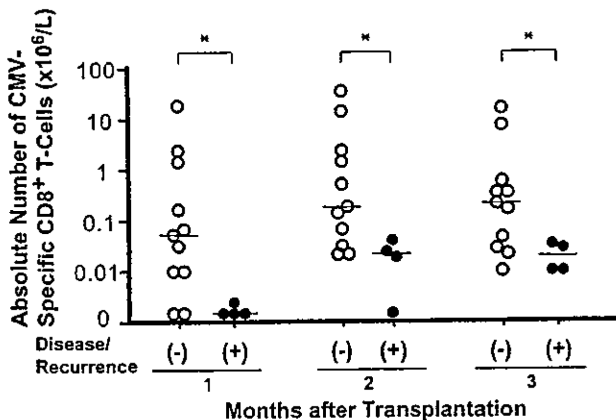

Figure 5. Reconstitution of cytomegalovirus (CMV)-specific CD8+ T-cells in patients who developed CMV disease or recurrent CMV reactivation following allogeneic stem cell transplantation. O indicates patients who did not develop CMV disease or recurrent CMV reactivation; ●, patients who developed CMV disease or recurrent CMV reactivation. Horizontal bars indicate median values (*, $P < .05$).

CD34-selected autologous transplantation delays immune reconstitution and causes an increased incidence of infectious complications [33-36]. An increased incidence of CMV disease has been reported in patients who received CD34-selected autologous transplantation [33,34,37]. In the analysis of 31 CMV-seropositive patients who received CD34-selected autologous PBSCT, Holmberg et al [37] reported that 7 patients (22.6%) developed CMV disease and that 4 (12.9%) died from the complications of their infection. In the present study, the reconstitution of CMV-specific CD8+ T-cells in CD34-selected autologous transplantation was delayed, a response similar to that of patients who developed grade II to IV acute GVHD or received ≥2 mg/kg corticosteroids. Infection surveillance, diagnostic work-up, and prevention strategies similar to those used with allogeneic transplant recipients may be required in CD34-selected autologous PBSCT recipients, as Crippa et al have recommended [34].

In the present study, the HLA-A*2402 QYDPVAALF tetramer was used to evaluate 1 component of the total CMV-specific T-cell immune response [18,27]. A multivariate analysis to identify factors influencing immunologic recovery against CMV could not be performed because of the limited number of patients in this study. However, our observations suggest that the detection of CMV-specific T-cells with the HLA-peptide tetramer is useful to assess immune reconstitution against CMV and to identify patients at risk for CMV disease or recurrent CMV reactivation after SCT.

Acknowledgment

We are grateful to Eiji Tashiro, Otsuka Tokyo Assay Laboratories, for expert technical assistance.

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Stem Cell Transplantation

Reduced-intensity allogeneic stem cell transplantation for renal cell carcinoma: *In vivo* evidence of a graft-versus-tumor effect

We report the cases of 3 patients with advanced renal cell carcinoma who underwent reduced-intensity allogeneic stem cell transplantation. In 2 partial responders, histologic analyses of metastases revealed prominent accumulation of CD8+ T cells and degenerative changes of clear cell carcinoma, suggestive of induction of tumor-specific cytotoxic T lymphocytes.

haematologica 2004; 89:375-376
(http://www.haematologica.org/2004/3/375)

Recently, reduced-intensity allogeneic stem cell transplantation (RIST) has been introduced into the treatment of renal cell carcinoma (RCC).[1-5] We report the preliminary results of RIST in 3 patients (Table 1) with advanced RCC refractory to cytokine-based therapy, and show the histologic analyses before and after transplantation. The patients and donors gave written informed consent to participate in this institutionally approved investigational protocol. The preparative regimen, consisting of cyclophosphamide and fludarabine, was entirely based on a previously reported study and included cyclosporine (CSP).[1] Patients received granulocyte colony-stimulating factor-mobilized peripheral blood stem cells from their HLA-identical siblings on day 0. Following transplantation, the degree of donor-recipient chimerism in both myeloid and T-cell lineages was assessed by polymerase-chain reaction assay according to a published method.[1]

All three patients achieved sustained myeloid and platelet engraftment with the proportion of donor cells in the peripheral blood exceeding 80% for both T cells and granulocytes within 2 months. We observed 2 partial responses in patients #1 and #3 six months and eight months after transplantation respectively: one response occurred after the development of chronic graft-versus-host disease (GVHD) and the other after acute GVHD: both coincided with full donor T-cell chimerism. Thereafter, the disease in patient #1 remained stable while GVHD responded to treatment with low-dose CSP plus steroids. Unfortunately, this patient died of bacterial pneumonia on day 554. In patient #3, chronic GVHD of the skin, salivary glands, and lung required treatment with CSP and steroids. Twenty months after transplantation, metastases started to grow despite a lack of change in GVHD. Reducing CSP and steroids caused acute respiratory failure due to chronic lung GVHD, though some regression of RCC metastases was observed. This

Table 1. Characteristics of the patients and outcome of transplantation.

Patient no.	*Age (yr)/sex*	*Histology*	*Sites of metastases*	*No. of previous systemic treatments*	*Nephrectomy*	*Age (yr)/sex of donor*	*CD34+ cells kg infused (×10⁶)*	*No. of CD3+ cells/kg infused (×10⁸)*	*GVHD*	*Response*	*Outcome*
1	64/M	Clear cell	Lung, pleura, bone, nodes	3	Yes	69/M	5.6	3.0	Extensive chronic skin, oral, salivary	PR	SD; died of pneumonia on day 554
2	58/F	Papillary	Pleura, liver, adrenal, nodes	2	No	59/M	9.7	4.3	Acute grade 3 skin, liver, GI	PD	Died of disease progression on day 68
3	56/M	Clear cell	Bone, lung, pleura, adrenal, nodes disease	3	Yes	51/F	4.9	2.4	Acute grade 2 skin, GI, extensive chronic skin, salivary, lung	PR	Died of progressive and GVHD on day 709

M: male; F: female; GVHD: graft-versus-host disease; GI: gastrointestinal; PR: partial response; SD: stable disease; PD: progressive disease.

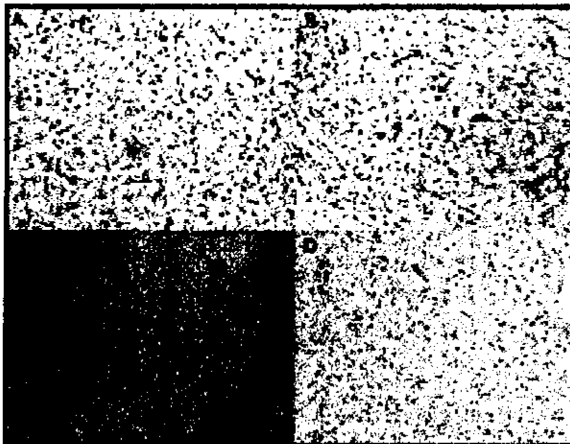

Figure 1. Photomicrographs of pleural metastases of RCC from patient #1. (A) Before transplantation, the clear cell architecture was intact in a pleural section obtained by open-lung biopsy. (B) In the pleural section taken post-mortem, tumor cell detritus and heavy mononuclear cell infiltration were seen. Immunohistochemical staining with monoclonal antibodies against CD8 (C) and CD4 (D) antigens showed that mononuclear cells in the post-mortem pleural section were primarily CD8⁺. Original magnification, ×200.

patient died of respiratory failure from GVHD and disease progression on day 709. Since the median survival of patients with cytokine therapy-resistant metastatic RCC is less than 6 months,[6] having stabilized disease progression for 12 months in patient #3 may be noteworthy. Patient #2 progressed soon after the transplantation and did not respond to withdrawal of CSP on day 18. To induce a graft-versus-tumor (GVT) effect, she received interleukin-2 plus donor-lymphocyte infusion with 3.0×10^{8} $CD3^{+}$ cells/kg on day 27. On day 45, grade III GVHD of the liver and intestine developed. Shortly thereafter lymphadenopathy in the neck and axilla regressed gradually, but there was no reduction in massive pleural effusions. Patient #2 died of disease progression on day 68.

Histologic analyses of the pleural metastasis in patient #1 revealed intact tumor cell architecture and the absence of lymphocyte infiltration before transplantation (Figure 1A). However, after transplantation we found an accumulation of mononuclear cells corresponding to a degenerative lesion of clear cell carcinoma (Figure 1B), which mainly consisted of $CD3^{+}CD8^{+}$ cells (Figure 1C), but not $CD4^{+}$ cells (Figure 1D). The other metastases in lung, bone, and lymph nodes also showed considerable penetration by infiltrating $CD8^{+}$ cells. These findings were also seen in patient #3. Post-mortem sections of bone, lung, pleura, adrenal, and lymph node metastases were found to contain abundant infiltrating $CD8^{+}$ cells. In patient #2, conversely, lymphocyte infiltration and destruction of tumor were not present in metastatic sites examined post-mortem, except for a small number of lymphocytes seen in the supraclavicular lymph node and the primary lesion of the left kidney as well as minimal destruction of the primary tumor.

Our study showed that the GVT effect was closely associated with infiltration of $CD8^{+}$ cells without infiltration of $CD4^{+}$ cells, although there is a possibility that T cells seen after death may differ from those at the time of response.

These findings suggest that the GVT effect after RIST may be mediated by induction of tumor-specific cytotoxic T lymphocytes (CTL) rather than induction of lymphocytes which secrete cytokines locally, since cytokine secretion and cytotoxicity are mainly functions of $CD4^{+}$ cells and $CD8^{+}$, cells respectively. The future direction of this study will be to isolate tumor antigens exclusively or preferentially presented by tumor cells, and generation of CTL specific for these tumor antigens.

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Key words: reduced-intensity stem cell transplantation, renal cell carcinoma, graft-versus-tumor effect, tumor-infiltrating lymphocytes.

Funding: this research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare, Japan.

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Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria–type cells

Xingmin Feng, Tatsuya Chuhjo, Chiharu Sugimori, Takeharu Kotani, Xuzhang Lu, Akiyoshi Takami, Hiroyuki Takamatsu, Hirohito Yamazaki, and Shinji Nakao

To identify candidate antigens in aplastic anemia (AA), we screened proteins derived from a leukemia cell line with serum of an AA patient and identified diazepam-binding inhibitor-related protein 1 (DRS-1). Enzyme-linked immunosorbent assay (ELISA) revealed high titers of anti–DRS-1 antibodies (DRS-1 Abs) in 27 (38.0%) of 71 AA patients displaying increased paroxysmal nocturnal hemoglobinuria (PNH)–type cells (PNH$^+$), 2 (6.3%) of 32 PNH$^-$ AA patients, 5 (38.5%) of 13 PNH$^+$ myelodysplastic syndrome (MDS) patients, and none of 42 PNH$^-$ MDS patients. DRS-1 gene was abundantly expressed in myeloid leukemia cell lines and in CD34$^+$ cells derived from healthy individuals. Stimulation of T cells from an AA patient displaying high DRS-1 Abs with a putative CD4$^+$ T-cell epitope (amino acid residues [aa's] 191-204) presented by HLA-DR15, which overlapped with a hot spot (aa's 173-198) of DRS-1 Ab epitopes, gave rise to T cells cytotoxic for L cells (murine fibroblasts) that were transfected with DRB1*1501 and DRS-1. Enzyme-linked immunospot assay demonstrated increased frequency of T-cell precursors specific to the DRS-1 peptide in other HLA-DR15$^+$ AA patients displaying high DRS-1 Ab titers. These findings indicate that DRS-1 may serve as an autoantigen eliciting immune attack against hematopoietic stem cells in a subset of AA patients characterized by increased PNH-type cells. (Blood. 2004;104:2425-2431)

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Introduction

Acquired aplastic anemia (AA), a bone marrow failure syndrome characterized by pancytopenia and bone marrow hypoplasia, has been the subject of study by hematologists for many years, as more than 70% of AA patients improve under immunosuppressive therapies such as antithymocyte globulin (ATG) and cyclosporine (CsA).[1-3] The dramatic effects of such T-cell suppressants on in vivo hematopoiesis suggest that immune system attack against hematopoietic stem cells plays an essential role in the development of AA.[4-6] However, despite extensive efforts to clarify the immune mechanisms of AA, the key antigens provoking immune response against hematopoietic stem cells remain unknown. This is largely due to a lack of animal models and the heterogeneity of pathogenesis in AA. Lack of good progenitor cell assays in humans has also hindered the elucidation of immune mechanisms in AA.

In organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM) and multiple sclerosis where autoreactive T cells play a primary role in pathogenesis, autoantibodies against target proteins of the pathogenic T cells are often detected.[7-10] Although such antibodies do not usually contribute to the pathogenesis of T-cell–mediated diseases, detection of the antibodies may prove useful in both identifying autoantigens and diagnosing immune mechanisms underlying the diseases.[11] We recently demonstrated that HLA-DRB1*1501 and increased paroxysmal nocturnal hemoglobinuria (PNH)–type cells represent prognostic markers for the immune mechanisms of AA.[12,13] Extensive investigation of antibodies in the sera of patients possessing HLA-DRB1*1501 and a minor population of PNH-type cells may be useful in identifying novel autoantigens in AA. Using immunofluorescent analysis, we previously found that antibodies to UT-7, a megakaryoblastic cell line, are frequently detectable in sera of AA patients who display increased PNH-type cells (PNH$^+$ patients; unpublished observation, T.C. and S.N., May 2001). These antibodies may recognize antigens that elicit T-cell responses against hematopoietic stem cells, allowing expansion of PNH-type stem cells.[14,15]

To examine these hypotheses, we screened proteins derived from UT-7 cDNA library using serum from a PNH$^+$ patient with HLA-DRB1*1501. Serologic identification of antigens by recombinant expression cloning (SEREX) analysis identified diazepam-binding inhibitor-related protein 1 (DRS-1) as an autoantigen that raises both antibody production and T-cell responses to antigen-presenting cells transfected with DRS-1 gene.

Patients, materials, and methods

Study subjects

Sera or plasma were obtained from 103 patients with AA (45 with severe AA and 58 with moderate AA); 55 patients with myelodysplastic syndrome

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Submitted May 13, 2004; accepted June 11, 2004. Prepublished online as *Blood* First Edition Paper, June 24, 2004; DOI 10.1182/blood-2004-05-1839.

Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports, and Culture of Japan (KAKENHI 15390298) and grants from the Research Committee for Idiopathic Hematopoietic Disorders, the Ministry of Health, Labor, and Welfare, Japan.

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(MDS), consisting of 46 with refractory anemia (RA) and 9 with refractory anemia with excess of blasts (RAEB); 5 patients with florid PNH; and 52 healthy individuals. Samples were cryopreserved at −80°C until use. All patients and controls provided informed consent according to the Declaration of Helsinki before supplying samples. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science.

AA and MDS were diagnosed in patients at Kanazawa University Hospital and other hospitals taking part in the bone marrow failure study group led by the Ministry of Health, Labor, and Welfare of Japan. MDS was diagnosed on the basis of cytopenia in peripheral blood, hypercellularity or normocellularity in the sternal or iliac bone marrow, and presence of dysplasia in at least 2 lineages of bone marrow cells. Cytogenetic abnormalities such as trisomy 8 and del(20)(q11) were noted in 14 of 46 RA patients and in 1 of 9 RAEB patients.

Detection of PNH-type cells

Percentages of $CD55^-CD59^-$ cells in $CD11b^+$ granulocytes and in glycophorin A^+ red blood cells (RBCs) were determined using 2-color flow cytometry as described previously.[12,16,17] Based on analytical results from 68 healthy individuals, presence of more than 0.003% $CD11b^+$ granulocytes and 0.005% glycophorin A^+ RBCs was considered abnormal.[12,17] Both thresholds exceeded the mean + 4 standard deviation (SD) for PNH-type granulocytes (0.0025%) and RBCs (0.0032%) determined on healthy individuals. Of the 103 AA patients, 71 (68.9%) displayed PNH-type cells ranged from 0.005% to 6.09%. The percentage of PNH-type cells was 0.005% to 0.01% in 7 (9.9%) patients, 0.01% to 0.1% in 22 (31.0%) patients, 0.1% to 1.0% in 32 (45.1%) patients, and 1.0% to 6.09% in 10 (14.1%) patients. Thirteen of the 46 (28.3%) RA patients displayed increased PNH-type cells, whereas none of the 9 RAEB patients did.

Preparation of cDNA library and SEREX

Poly(A) RNA was purified from UT-7 cells (kindly provided by Dr N. Komatsu of Jichi Medical School, Japan), and a cDNA expression library was constructed with a λZAPII expression vector using a cDNA library kit (Stratagene, La Jolla, CA). Screening for antigens recognized by autoantibodies in the patient's serum was performed as described previously.[18] Briefly, XL1-Blue *Escherichia coli* (Stratagene) was transformed with recombinant phages, plated on agar at 5×10^4 plaques per 150-mm Petri dish, and cultured at 37°C for 6 to 8 hours. Expression of recombinant proteins was induced by incubating bacterial lawns with an overlay of iso-propyl β-D-thiogalactoside (IPTG; Promega, Madison, WI) saturated nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Transfer of released proteins was allowed to proceed for an additional 4 hours at 37°C. Membranes were then washed with 25 mM tris(hydroxymethyl)aminomethane (Tris)–buffered solution (TBS) containing 150 mM NaCl, 2.5 mM KCl, and 0.05% Tween 20 (TBST; pH 7.5) to remove excess agar and blocked overnight with 5% nonfat dry milk in TBS at 4°C.

Serum was obtained from an untransfused 71-year-old AA patient (patient 1) who demonstrated CsA-dependent recovery of hematopoiesis and displayed an increase in PNH-type cells.[19] HLA-DRB1 alleles in this patient included 1501 and 0405. Serum was preabsorbed with bacterial lysates to minimize nonspecific antibody binding. Membranes were then incubated with the serum diluted at 1:200 in TBS containing 1% bovine serum albumin (BSA/TBS). Specific binding of immunoglobulin G (IgG) antibodies to recombinant proteins expressed on the lytic plaques was detected by incubating the membranes with alkaline phosphatase-conjugated antihuman IgG antibody (1:2000; Jackson ImmunoResearch, West Grove, PA). Antigen-antibody complexes were visualized by adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP; KPL, Guildford, United Kingdom) and nitroblue tetrezolium (NBT; KPL). cDNA inserts from reactive clones were subcloned to monoclonality, excised in vivo to the pBluescript SK(−) phagemid (Stratagene), and sequenced using an ABI PRISM3100 sequencer (PE Applied Biosystems, Foster, CA).

Purification of bacterially expressed fusion proteins and Western blotting

Full-length DRS-1 cDNA obtained from SEREX analysis was subcloned into the pET-44a (+) vector (Novagen, Madison, WI) for expression of a His-tag fusion protein. Synthesized proteins were purified using a His bind kit (Novagen) according to the manufacturer's instructions. A His-tag encoded by pET-44a (+) without the insert was also purified for use as a negative control. Native DRS-1 protein was released from His-tag DRS-1 protein using a thrombin cleavage kit (Novagen). Size of the recombinant proteins was confirmed by Western blotting using mouse anti-His monoclonal antibody (mAb; Amersham Pharmacia Biotech, Piscataway, NJ) as described previously.[20] To detect specific antibodies in serum, 1:200 diluted serum was incubated with blotted membranes.

Enzyme-linked immunosorbent assay (ELISA)

Each well of a 96-well Nunc-Immuno plate (Nalge-Nunc International, Roskilde, Denmark) was covered with 100 μL of coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) containing 1 μg/mL of purified recombinant DRS-1 protein and kept overnight at 4°C. Plates were washed and covered with phosphate-buffered saline (PBS) containing 10% fetal calf serum overnight at 4°C to block nonspecific binding of serum protein to DRS-1. Sera from patients were added to a final dilution of 1:1000 and incubated at room temperature for 1 hour. After washing, plates were incubated with peroxidase-conjugated goat antihuman IgG antibody (1:10 000; Jackson ImmunoResearch) at room temperature for 1 hour. Finally, plates were washed and incubated with 3,3',5,5'-tetramethylbenzidine substrate (Pierce, Rockford, IL) at room temperature for 30 minutes, and the optic density (OD) absorbance at 450 nm was read using an SLT EAR 340AT ELISA reader (SLT-Labinstruments, Grödig, Austria). A positive reaction was defined as an absorbance value exceeding the mean + 2 SDs for the OD absorbance value of sera from the 52 controls.

Cell lines

A chronic myeloid leukemia cell line KH88 was kindly provided by Dr M. Yasukawa of Ehime University. K562, KU812, Daudi, U937, HEL, and Molt-4 were purchased from RIKEN BRC (Ibaraki, Japan). A murine fibroblast cell line, L cell, transfected with HLA-DRB1*1501 (1501-L cell) or with HLA-DRB1*0101 (0101-L cell) was provided by Dr Y. Nishimura of Kumamoto University. Lymphoblastoid cell lines (LCLs) were established from peripheral blood mononuclear cells (PBMCs) of 7 healthy individuals using B95-8 (ATCC, Manassas, VA).

Ribonuclease protection assay (RPA)

RNA probes were prepared for detecting gene expression of DRS-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by amplifying a cDNA fragment of DRS-1 and that of GAPDH using specific primer sets (sense primer 5'-CTATTCGATGCCGTGTATGC-3', and antisense primer 5'-GCCTGGTCCAGACTTCTTTC-3' for DRS-1; sense primer 5'-TGAACGGGAAGCTCACTGGC-3', and antisense primer 5'-AGGTC-CACCACCCTGTTGCT-3' for GAPDH) followed by subcloning into a pGEM-T Easy vector (Promega). Linearized plasmid DNA containing DRS-1 or GAPDH by cutting with *Sal*I were used as templates to synthesize biotin-labeled RNA probes using MAXIscript T7 kit (Ambion, Austin, TX) with minor modifications. A total of 10 ng of the RNA probe was used for hybridization with 20 μg total RNA of each kind of cell line. RPA was performed using the Ribo-Quant RPA kit (PharMingen, San Diego, CA), and chemiluminescent signals were detected using the Non-Rad Detection kit (PharMingen) according to the manufacturer's instructions.

Isolation of $CD34^+$ cells and $CD4^+$ T cells

$CD34^+$ cells were isolated from the bone marrow of 3 healthy volunteers using a CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD4^+$ T cells were separated from cultured T cells using Dynabeads M-450 CD4 (Dynal Biotech ASA, Oslo, Norway).

Quantification of DRS-1–specific mRNA with real-time polymerase chain reaction (PCR)

Total RNA was extracted from PBMCs, $CD34^+$ cells of healthy volunteers, and leukemia cell lines using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of 1 μg of RNA into cDNA was performed using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and stored at −20°C until use. Quantification of DRS-1 gene expression was performed using a LightCycler (Roche Diagnostics, Tokyo, Japan) with specific primers described under "Ribonuclease protection assay (RPA)." The LightCycler with a GAPDH primer kit (Search LC, Heidelberg, Germany) was used for quantification of mRNA for GAPDH, a housekeeping gene, in the same samples. The relative amount of DRS-1 mRNA to GAPDH mRNA (DRS-1/GAPDH) was used to represent expression level of the DRS-1 gene.

Epitope mapping

In order to detect hot spots of epitopes recognized by DRS-1 Abs of different patients, epitope mapping was performed using the NovaTope library construction and screening system (Novagen). Briefly, DRS-1 cDNA was randomly digested using DNase I into small fragments of 50 to 150 bp. After both ends were blunted and a single 3′-deoxyadenosine residue added, fragments were ligated into the pSCREEN T-Vector. NovaBlue (DE3)–competent cells were transformed using the constructed pSCREEN T-Vector for peptide expression. Colonies were transferred onto nitrocellulose filters and lysed in a chloroform vapor chamber. After denaturation and blocking, filters were incubated with sera from AA patients possessing DRS-1 Abs overnight at 4°C, then immunodetection was performed by incubating membranes with alkaline phosphatase–conjugated antihuman IgG antibodies (1:2000; Jackson ImmunoResearch). Antigen-antibody complexes were visualized by adding BCIP and NBT. Positive colonies were selected, and plasmid DNA was extracted from the colonies using a miniprep DNA purification system (Promega). Proteins derived from positive clones were purified using a His bind kit (Novagen) as described under "Purification of bacterially expressed fusion proteins and Western blotting."

Preparation of peptides as an epitope candidate within DRS-1

Peptide sequences within DRS-1 that can be presented by HLA-DR15 were deduced based on the TEPITOPE algorithm[21] with a prediction threshold (ie, percentage of best-scoring natural peptides) of 5%. Two positive peptides, amino acid residues (aa's) 191-204 (AVLLREFVGCFIDF, peptide 1) and aa's 351-364 (TNAVVNFLSRKSKL, peptide 2), as well as a negative peptide, aa's 95-108 (SSQVEPGTDRKSTG, peptide 3), which was predicted to display no binding to the HLA-DR15 molecule, were synthesized using a Rainin Symphony multiple-peptide synthesizer (Rainin, Woburn, MA). Synthetic peptides were lyophilized, reconstituted in dimethyl sulfoxide at 5 mg/mL, and diluted in RPMI 1640 (Gibco, Grand Island, NY) as needed.

Transduction of L cells with DRS-1 gene

The DRS-1 minigene was amplified by PCR using pBluescript SK(−) harboring full-length DRS-1 cDNA as a template. The primer set used for PCR included 5′-GGGCTCGAGCCCGCCGCCACCATGCTGACT-AACTTCACTGATATT-3′ and 5′-GCGGCCGCTCACAGTTTTGATTT-TCTGGATAA-3′ appended *Xho*I and *Not*I sites (underlined sequences) at the 5′ and 3′ ends of the DRS-1 minigene, respectively. PCR products were inserted into a pGEM-T Easy vector (Promega). After propagation, DRS-1 minigene was released by digestion with *Xho*I and *Not*I, then subcloned into pCAGIPuro vector (kindly provided by Dr H. Niwa, RIKEN). The pCAGIPuro vector harboring the DRS-1 minigene was used to transfect 1501- or 0101-L cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable transfectants were obtained by selection using RPMI 1640 medium containing puromycin (5 μg/mL; BD Biosciences Clontech, Palo Alto, CA).

Establishment of DRS-1–specific T cells and ^{3}H-thymidine incorporation assay

PBMCs obtained from an AA patient (patient 2) with HLA-DR15 displaying high titers of DRS-1 Abs were cultured for 14 days with irradiated 1501-L cells that were pulsed with DRS-1 peptides, and $CD4^+$ T cells were separated. Proliferative responses of cultured $CD4^+$ T cells to 1501-L cells or 0101-L cells transfected with the DRS-1 gene were measured using ^{3}H-thymidine incorporation assay. A total of 5×10^4 $CD4^+$ T cells were cultured in 96-well U-bottomed plates (IWAKI, Chiba, Japan) with the same number of 45 Gy-irradiated L-cell transfectants. After 3 days of incubation, 1 μCi (0.037 MBq) of ^{3}H-thymidine (6.7 Ci/mmol [2.48×10^{11} Bq/mmol]; Dupont NEN Products, Boston, MA) was added to each well. Cultured cells were harvested after 6 hours, and ^{3}H-thymidine incorporation was measured. Data were represented as relative proliferative index calculated as ^{3}H-thymidine incorporation by T cells cultured with L-cell transfectants relative to ^{3}H-thymidine incorporation by T cells cultured without L-cell transfectants.

^{51}Cr-release assay

L-cell transfectants were incubated with 100 μCi (3.7 MBq) of ^{51}Cr at 37°C for 1 hour after washing with PBS. Labeled cells were washed 3 times with PBS and suspended in complete medium containing 10% pooled human AB serum. Labeled cells (5×10^3) were incubated with various numbers of DRS-1–specific $CD4^+$ T cells for 4 hours. The release of ^{51}Cr into medium was measured using a γ-counter. Percentage of specific lysis (mean ± SD) obtained in the ^{51}Cr-release assay was determined from triplicate cultures as follows: 100 × (experimental release cpm − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm), where cpm indicates counts per minute.

Determination of T-cell precursor frequencies specific to DRS-1 peptides

Approximately 2×10^6 PBMCs were cultured for 7 days in RPMI 1640 supplemented with heat-inactivated human serum (10%) and L-glutamine (2 mM) containing 20 μg/mL of a DRS-1–derived peptide. On day 7, 2×10^4 1501-L cells were pulsed with 20 μg/mL of the same peptide. After a 4-hour incubation, L cells were washed twice with RPMI1640, irradiated, and added to the cultured PBMCs. Interleukin 2 (IL-2) was added on day 8 at 100 U/mL. On day 14 of culture, $CD4^+$ T cells were separated and subjected to enzyme-linked immunospot assay (ELISPOT) using an interferon γ (IFN-γ) ELISPOT assay kit (BioSource International, Camarillo, CA). Briefly, 10^5 induced $CD4^+$ T cells were transferred into each well of the ELISPOT plate then cocultured with 2×10^4 peptide-pulsed L cells overnight. IFN-γ spots were then detected according to the manufacturer's instructions.

Statistics

Differences in prevalence of DRS-1 Ab titers in serum among different patient groups and in responses of DRS-1–specific T cells to different target cells were examined using Fisher exact test and Student *t* test, respectively. The logistic procedure was used to identify factors significantly associated with a good response to immunosuppressive therapy (IST).

Results

Identification of cDNA clones recognized by serum of an AA patient

SEREX analysis using the UT-7 cDNA library and diluted serum from an AA patient (patient 1) identified 6 independent clones including DRS-1, *Homo sapiens* KIAA0907 protein, α1HB subunit of voltage-dependent T-type calcium channel, U2 small nuclear ribonucleoprotein auxiliary factor 35-kDa subunit related-protein 2, hemoglobin γ-1 chain, and lens epithelium-derived growth factor. When sera from another 10 AA patients were screened for

antibodies to these clones using a phage plate assay, only the DRS-1 clone was recognized in 2 patients. Focus was thus placed on DRS-1 for further studies.

Detection of specific antibodies to DRS-1 in AA patients

To confirm the presence of antibodies specific to DRS-1 in the sera of AA patients, a recombinant His-tag DRS-1 protein was prepared in addition to His-tag and DRS-1 proteins. Figure 1A shows the results of Western blotting using these recombinant proteins. Serum of a PNH$^+$ AA patient displayed both His-tag DRS-1 and DRS-1, but not His-tag, whereas serum of a healthy individual did not display any of these recombinant proteins (Figure 1A).

Figure 1B shows results of the same Western blotting for selected patients with AA or MDS and healthy individuals. Clear bands indicating the presence of DRS-1 Abs were produced by the sera of several PNH$^+$ AA patients but not by the sera of AA patients without PNH-type cells (PNH$^-$ AA patients) or those of PNH$^-$ MDS patients.

Measurement of DRS-1 Ab titers with ELISA in patients with AA and MDS

To measure titers of DRS-1 Abs in serum, we established an ELISA using recombinant DRS-1 protein. Figure 2 shows antibody titers in the sera of different groups of patients. AA and MDS patients were divided into 2 groups based on the presence of increased PNH-type cells, which represent a marker for immune pathophysiology in AA.[12,13] Twenty-seven (38.0%) of the 71 PNH$^+$ AA patients and 5 (38.5%) of 13 PNH$^+$ MDS patients showed antibody titers greater than the cutoff value, which were significantly higher than that of PNH$^-$ AA (6.3%, 2 of 32) and PNH$^-$ MDS (0 of 42) patients, but there was no significant difference between PNH$^+$ AA and PNH$^+$ MDS patients (P = .976). All 5 MDS patients with DRS-1 Abs had RA without karyotypic abnormalities. None of 5 patients with florid PNH were positive for DRS-1 Abs (data not shown).

Response to IST in AA patients with DRS-1 Abs

To determine if the presence of DRS-1 Abs reflects immune pathophysiology in AA, we selected 22 patients whose sera were tested for DRS-1 Abs before IST and compared response rates to IST between DRS-1 Ab$^+$ patients and DRS-1 Ab$^-$ patients. Response to IST was evaluated at 6 months after therapy according to the response criteria of Camitta.[22]

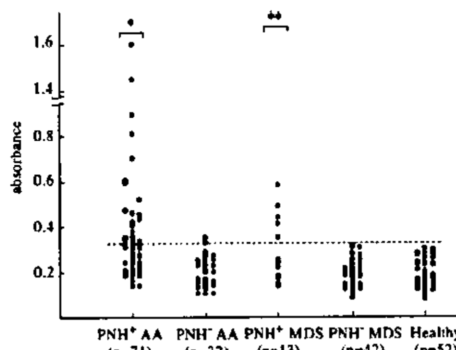

Figure 2. Titration of DRS-1 Abs in sera of patients using ELISA. Antibody titers against purified His-tag DRS-1 in the sera of 71 PNH$^+$ AA patients, 32 PNH$^-$ AA patients, 13 PNH$^+$ MDS patients, 42 PNH$^-$ MDS patients, and 52 healthy individuals were determined using sera diluted at a 1:1000 dilution. The dotted line denotes a cutoff value defined as mean + 2 SD for absorbance in 52 healthy individuals. Asterisks indicate a prevalence of DRS-1 Ab titers significantly higher than that of PNH$^-$ AA patients, PNH$^-$ MDS patients, and healthy individuals (*P < .001, **P < .05).

All 11 (100%) DRS-1 Ab$^+$ patients responded to ATG + CsA (4) or CsA (7), whereas 6 (55%) of the 11 DRS-1 Ab$^-$ patients improved with ATG + CsA (4) or CsA (2). Several factors that may influence response to IST were analyzed using multivariate analysis. The presence of DRS-1 Abs (P = .0026) was significantly associated with a good response to IST, whereas age (P = .2439), sex (P = .3852), severity of AA (P = .4159), and increased PNH-type cells (P = .7389) did not affect response to IST in the 22 patients.

Expression of DRS-1 gene by hematopoietic cells

Although DRS-1 gene expression is reportedly ubiquitous, expression of the gene by hematopoietic cells has not been studied in detail. We studied DRS-1 gene expression in various leukemia cell lines using RPA. Myeloid leukemia cell lines such as K562, UT-7, KU812, and KH88 displayed high expression of the DRS-1 gene (Figure 3A). Conversely, lymphoid or monocytoid leukemia cell lines did not display detectable levels of DRS-1 mRNA. When expression of the DRS-1 gene was quantified using real-time PCR, DRS-1/GAPDH ratios of K562, UT-7, and bone marrow CD34$^+$

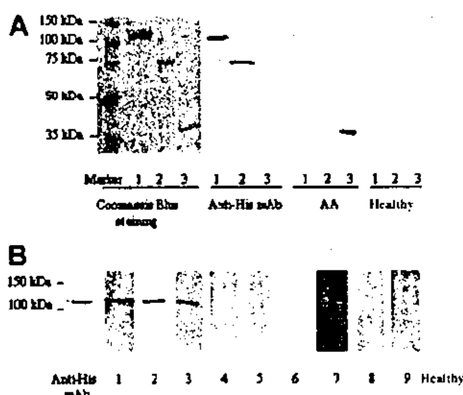

Figure 1. Specific antibody to recombinant DRS-1 in the serum of an AA patient. (A) Purified His-tag DRS-1, His-tag, and native DRS-1 were loaded in lanes 1, 2, and 3, respectively. Proteins were visualized using Coomassie blue staining. Blotted membranes were incubated with anti-His mAb, serum of a PNH$^+$ AA patient, and serum of a healthy individual for detection of DRS-1 Abs. (B) An equal amount of purified His-tag DRS-1 protein was used to detect antibodies specific to DRS-1 in sera from 3 PNH$^+$ AA patients (lanes 1-3), 3 PNH$^-$ AA patients (lanes 4-6), 3 PNH$^-$ MDS patients (lanes 7-9), and a healthy individual (Healthy).

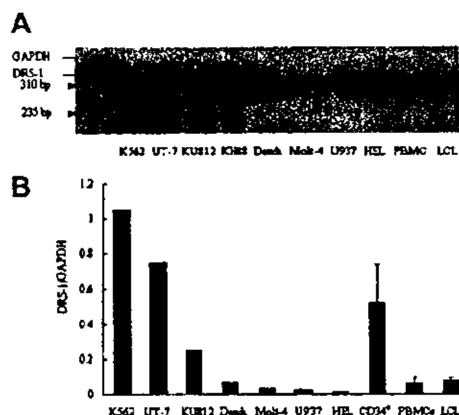

Figure 3. DRS-1 gene expression in hematopoietic cells. (A) Total RNA (20 μg) from each cell line was subjected to ribonuclease protection assay using biotin-labeled DRS-1 RNA probe and GAPDH RNA probe. The protected GAPDH probe and DRS-1 probe were visualized at 310 bp and 235 bp, respectively. (B) The same amounts of cDNA derived from each cell line or CD34$^+$ cells were used to amplify DRS-1 or GAPDH, respectively. Relative expression levels of DRS-1 to GAPDH were determined as DRS-1/GAPDH. The levels for CD34$^+$, PBMCs, and LCLs represent mean + SD of 3, 6, and 7 healthy individuals, respectively.

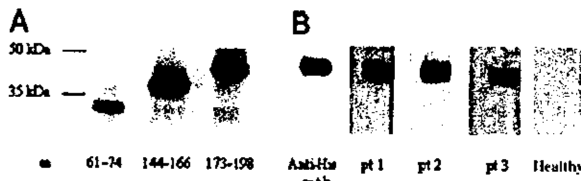

Figure 4. Mapping of antibody epitopes in DRS-1 protein. (A) Lysates of transformed *E coli* expressing 3 different DRS-1 fragments (aa's 61-74, aa's 144-166, aa's 173-198) were tested for reactivity to the original serum from patient 1 (pt 1). (B) Recombinant proteins derived from one epitope clone aa's 173 to 198 were purified and subjected to Western blotting using the sera of AA patients who exhibited antibodies specific to the native DRS-1 protein. Patient 2 (pt 2) and patient 3 (pt 3) represent other PNH$^+$ AA patients who showed high titers of DRS-1 Abs.

cells from healthy individuals were 1.62, 0.75, and 0.51, respectively, which were 35-, 16-, and 11-fold higher than DRS-1/GAPDH ratios for PBMCs from healthy individuals (Figure 3B). Other leukemia cell lines such as Daudi, Molt-4, U937, and HEL, as well as LCLs derived from healthy individuals, displayed expression levels similar to those of normal PBMCs.

Antibody epitopes of DRS-1 protein

To determine whether there is a common epitope recognized by antibodies derived from different AA patients, randomly cut fragments of DRS-1 were ligated into pSCREEN T-Vector, and DRS-1 fragments derived from ligated plasmids were examined for reactivity to sera that were positive for DRS-1 Abs. Immunoblotting using the original serum revealed 3 antibody epitopes corresponding to aa's 61 to 74, aa's 144 to 166, and aa's 173 to 198 of DRS-1 (Figure 4A). Among these 3 epitopes, only aa's 173 to 198 were recognized by sera from other patients including patients 2 and 3 carrying HLA-DR15 (Figure 4B). Antibodies to this epitope were present in 7 (53.8%) of the 13 DRS-1 Ab$^+$ patients.

T-cell responses to endogenous DRS-1 protein

To determine if DRS-1 can elicit T-cell responses to antigen-presenting cells (APCs) expressing DRS-1, we looked for peptides that can be presented by HLA-DR15 in the aa sequence of DRS-1 and identified 2 putative CD4$^+$ T-cell epitopes, peptide 1 and peptide 2. Interestingly, peptide 1 (aa's 191-204) was found to overlap with the common antibody epitope aa's 173-198. PBMCs from patient 2 stimulated by peptide 1 were examined for reactivity to 1501-L cells transduced with the DRS-1 gene. Primed CD4$^+$ T cells exhibited significantly higher proliferative response to 1501-L cells transduced with DRS-1 gene than to nontransduced 1501-L cells or to 0101-L cells transduced with DRS-1 gene ($P < .05$; Figure 5A). DRS-1–specific CD4$^+$ T cells also killed DRS-1–transduced L cells in a dose-dependent fashion. Cytotoxicity against DRS-1–transduced 1501-L cells reached 52.8% at an effector-target ratio of 20, significantly higher than that against DRS-1–transduced 0101-L cells or nontransduced 1501-L cells ($P < .001$; Figure 5B). These findings suggest that DRS-1 can be processed in APCs, and the DRS-1 peptide presented by HLA-DR15 may provoke T cells to attack APCs expressing the DRS-1 gene.

T-cell precursors specific to DRS-1 peptides in peripheral blood of DRS-1 Ab$^+$ patients

In order to determine the frequency of T-cell precursors specific to DRS-1 peptides in peripheral blood T cells of DRS-1 Ab$^+$ AA patients, another peptide (peptide 3) with low affinity to HLA-DR15 was prepared, and PBMCs in patient 2 and patient 3 were examined using ELISPOT assay. Stimulation of PBMCs from patient 2 with peptide 1 and peptide 2 induced a higher number of INF-γ spots than stimulation with peptide 3 (Figure 6A). Such high induction of INF-γ spots was not induced in PBMCs of a DRS-1 Ab$^-$ AA patient (patient 4) and a healthy individual. Stimulation with 0101-L cells pulsed with peptide 1 and peptide 2 also failed to induce INF-γ spots from the same PBMCs. In patient 3, only peptide 1 induced as many as INF-γ spots as phorbol myristate acetate (PMA) plus ionomycin did (Figure 6B).

Discussion

DRS-1 was identified as a novel candidate for autoantigen in AA, using the SEREX method with a cDNA library derived from UT-7 and serum from an AA patient possessing increased PNH-type cells and HLA-DRB1*1501. Several findings in the present study suggest that DRS-1 is involved in the immune pathophysiology of AA. These include a high prevalence of DRS-1 Abs in PNH$^+$ AA and PNH$^+$ RA patients, high rate of response to immunosuppressive therapy in patients with this antibody, high expression of DRS-1 gene by myeloid leukemia cell lines and CD34$^+$ cells from healthy individuals, inducibility of specific T cells recognizing APCs that express the DRS-1 gene, and the presence of T-cell precursors specific to DRS-1 in peripheral blood from AA patients displaying DRS-1 Abs.

DRS-1 is identical to monofunctional peroxisomal Δ^3, Δ^2-enoyl-CoA isomerase (*PECI*).[23] ECI is unique in that its activity is essential for the β-oxidation of all unsaturated fatty acids,[24,25] and presence of this enzyme has been demonstrated in mammalian peroxisomes and mitochondria.[23] This gene is abundantly expressed in various tissues, including the heart, lung, brain, and liver, but not in peripheral blood leukocytes.[23] Expression of *PECI* may be increased in immature hematopoietic cells because they require active utilization of fatty acids as an energy source.[23] Indeed, the results of our RPA and real-time PCR indicate that *DRS-1* is highly expressed by immature myeloid cells, including CD34$^+$ cells, supporting our hypothesis that DRS-1 could represent a target antigen of immune system attack directed against hematopoietic progenitor cells in AA. Although DRS-1 is ubiquitously expressed, only hematopoietic progenitor cells may be vulnerable to

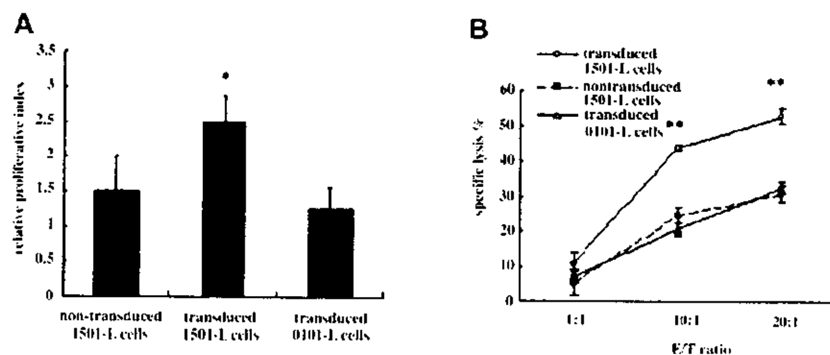

Figure 5. Response of DRS-1–specific T cells to APCs with DRB1*1501 expressing the DRS-1 gene and immature myeloid cells. (A) ^{3}H-thymidine incorporation of DRS-1–specific T cells to DRS-1–transduced 1501-L cells and 0101-L cells, as well as nontransduced 1501-L cells. Values represent mean + SD of triplicate cultures from 4 different experiments. (B) Cytotoxicity of DRS-1–specific T cells against DRS-1–transduced 1501-L cells and 0101-L cells, as well as nontransduced 1501-L cells. L-cell transfectants were incubated with differing numbers of DRS-1–specific T cells in a 4-hour cytotoxicity assay. Values represent mean ± SD of duplicate cultures from 3 different experiments. Asterisks in Figure 5A-B indicate values significantly different from nontransduced 1501-L cells or transduced 0101-L cells (*$P < .05$, **$P < .001$). E/T indicates effector-target ratio.

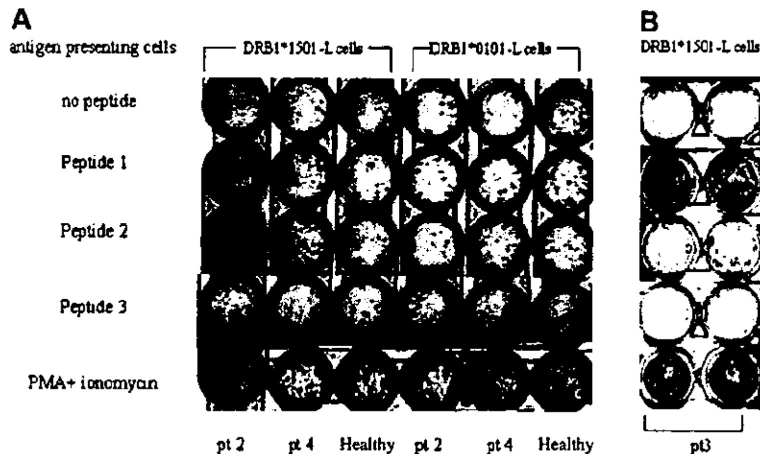

Figure 6. Frequency of T-cell precursors specific to the DRS-1 epitope. (A) PBMCs of a DRS-1 Ab^+ patient (pt 2), a DRS-1 Ab^- patient (patient 4; pt 4), and a healthy individual were subjected to ELISPOT assay using different combinations of APCs and DRS-1–derived peptides. (B) Another DRS-1 Ab^+ patient (pt 3) was subjected to ELISPOT assay. The figure shows results of duplicate culture.

cellular immune responses to DRS-1 due to the expression of functional HLA-DR molecules.[26]

Antibodies to DRS-1 have been detected in patients with autoimmune diabetes,[27] breast cancer,[28] renal cancer,[29] and hepatocellular carcinoma[30] using the SEREX method. However, the significance of antibodies in the pathophysiology of these diseases has remained unclear due to the low prevalence (≤ 8%) of antibodies in these diseases. ELISA in the present study revealed significantly higher titers of DRS-1 Abs compared with healthy controls in 38.0% of PNH^+ AA patients and 38.5% of PNH^+ MDS patients, both of which are considered to have immune-mediated bone marrow failure.[12,13,31] As for MDS patients, DRS-1 Abs were detected only in PNH^+ RA patients, supporting the significance of a small number of PNH-type cells as a marker for immune pathophysiology. Antibody production against DRS-1 is not a secondary phenomenon associated with destruction of PNH-type cells because none of 5 patients with florid PNH displayed DRS-1 Abs. In PNH^+ AA patients who do not show increased titers of DRS-1 Abs, antigens other than DRS-1 may be involved in immune pathogenesis of AA. Multivariate analysis identified presence of DRS-1 Abs as a factor predicting a good response to IST. PNH^+ bone marrow failure is thus the first disease where antibody response to DRS-1 has been implicated in an autoimmune pathophysiology.

Overlap of immunodominant T- and B-cell epitopes has been observed in pyruvate dehydrogenase complex for primary biliary cirrhosis,[32-35] myelin basic protein[36,37] and proteolipid protein for multiple sclerosis,[38-41] and glutamic acid decarboxylase 65 for IDDM,[42-44] suggesting that this is a common theme for autoimmune diseases. The hot spot of the antibody epitope we identified in epitope mapping was shared by 53.8% of DRS-1 Ab^+ AA patients, and the C-terminal half of the epitope sequence overlapped with a deduced $CD4^+$ T-cell epitope (aa's 191-204) presented by HLA-DR15. Colocalization of the immunodominant T- and B-cell epitopes of DRS-1 may be important in the development of autoimmune responses against DRS-1 in AA. Antigen-specific B cells play important roles as APCs by way of uptake of antigens via surface immunoglobulin.[45,46] Antibody binding to specific antigen modulates antigen processing by human B lymphoblastoid cells.[46] In antigen-presentation of tetanus toxoid, a single bound antibody or associated F(ab) fragment can enhance the presentation of one T-cell determinant while strongly suppressing the presentation of a different T-cell determinant. Both suppressed and boosted determinants fall within an extended domain of antigen stabilized by the antibody during proteolysis.[47]

Some intracellular proteins can be processed in the cytoplasm and chaperoned to HLA class II molecules by the invariant chain peptide.[48] Whether proteosome proteins like DRS-1 can take this pathway to be presented by HLA-DR is unknown. The specific response of DRS-1–specific T cells to HLA-DRB1*1501-L cells transfected with DRS-1 gene strongly suggests that the cytoplasmic protein can be processed by APCs and the cells can be targeted through recognition of the DRS-1 peptide–HLA-DR15 complex by specific T cells. Several studies have shown that HLA-DR molecules in hematopoietic progenitor cells bind some intracellular proteins such as tubulin β-chain, prolidase, thrombospondin 1, and granzyme.[49] ELISPOT assay in the present study demonstrated that T-cell precursors specific to peptide 1 were increased in 2 AA patients carrying HLA-DR15 and DRS-1 Abs. The DRS-1 epitope may thus stimulate T cells to raise both antibodies and $CD4^+$ T cells specific to DRS-1. Although we could not examine the effect of DRS-1–specific T cells on the growth of hematopoietic progenitor cells due to the unavailability of autologous $CD34^+$ cells, these findings suggest that DRS-1–specific $CD4^+$ T cells may contribute to the development of AA by directly killing hematopoietic progenitor cells. The high prevalence of immune response to DRS-1 in PNH^+ AA patients appears to support this hypothesis, as $CD4^+$ T cells specific to certain antigens on hematopoietic cells allow expansion of PNH clones.[14]

Hirano et al[50] recently identified kinectin as a possible antigen in AA, using the SEREX method with a cDNA library derived from fetal liver cells. They induced kinectin-specific $CD8^+$ T cells from PBMCs of HLA-A2–positive healthy donors and demonstrated that T cells inhibited in vitro growth of hematopoietic progenitor cells in an HLA-A2–restricted fashion. Although the number of AA patients they studied was very low, $CD8^+$ T cells specific to endogenous proteins like kinectin may conceivably play a role in bone marrow failure for some AA patients. However, the high incidence of HLA-DR15 and increased PNH-type cells in immune-mediated AA suggest the importance of $CD4^+$ T cells rather than $CD8^+$ T cells in the development of AA.[13] Our study demonstrated for the first time that immune responses to a protein abundantly expressed in hematopoietic progenitor cells by both T and B cells are operative in immune-mediated AA. Identification of DRS-1–specific T cells with HLA class II tetramers and subsequent functional analysis would further clarify the roles of DRS-1 in the pathogenesis of AA.

Acknowledgment

We would like to thank Dr Haruo Tsuchiya for his helpful discussion and ideas.

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Featured Article

Successful Engraftment After Reduced-Intensity Umbilical Cord Blood Transplantation for Adult Patients with Advanced Hematological Diseases

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ABSTRACT

Purpose: **The purpose of this research was to evaluate the feasibility of reduced-intensity unrelated cord-blood transplantation (RI-UCBT) in adult patients with advanced hematological diseases.**

Experimental Design: **Thirty patients (median age, 58.5 years; range, 20–70 years) with advanced hematological diseases underwent RI-UCBT at Toranomon Hospital between September 2002 and August 2003. Preparative regimen composed of fludarabine 25 mg/m^2 on days −7 to −3, melphalan 80 mg/m^2 on day −2, and 4 Gy total body irradiation on day −1. Graft-*versus*-host disease prophylaxis was composed of cyclosporin alone.**

Results: **Twenty-six patients achieved primary neutrophil engraftment after a median of 17.5 days. Median infused total cell dose was 3.1 × 10^7/kg (range, 2.0–4.3 × 10^7/kg). Two transplant-related mortalities occurred within 28 days of transplant, and another 2 patients displayed primary graft failure. Cumulative incidence of complete donor chimerism at day 60 was 93%. Grade II-IV acute graft-*versus*-host disease occurred in 27% of patients, with median onset 36 days. Primary disease recurred in 3 patients, and transplant-related mortality within 100 days was 27%. Estimated 1-year overall survival was 32.7%. Excluding 7 patients with documented infection, 19 patients displayed noninfectious fever before engraftment (median onset, day 9). Manifestations included high-grade fever, eruption, and diarrhea. The symptoms responded well to corticosteroid treatments in 7 of 13 treated patients.**

Conclusion: **This study demonstrated the feasibility of RI-UCBT in adults.**

INTRODUCTION

Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) is a curative treatment for refractory hematological malignancies. The therapeutic benefits are attributable to myeloablative radiochemotherapy and graft-*versus*-leukemia effects (1), whereas the severe regimen-related toxicity (RRT; Ref. 2) limited allo-HSCT to young patients without comorbidities.

Reduced-intensity stem-cell transplantation (RIST) using a nonmyeloablative preparative regimen has been developed to decrease RRT, whereas preserving adequate antitumor effects (3–5). Different pioneering conditioning regimens for RIST have been investigated, such as those including purine analogs (3–6) and total body irradiation (TBI). Although RIST has been attempted in various diseases (5, 6), suitable preparative regimens with adequate immunosuppression have yet to be established.

Although allo-HSCT from an HLA-identical sibling is promising, only 30% of the patients have an HLA-identical sibling donor. The value of unrelated cord-blood transplantation (UCBT) was confirmed for pediatric patients (7, 8). It has seen recent application in adult patients (9). Whereas the potential graft-*versus*-leukemia effects by cord-blood (CB) without severe graft-*versus*-host disease (GVHD; Ref. 10) has been reported, current questions include whether CB provides a sufficient number of stem cells for adults and suitable graft-*versus*-leukemia effects.

Reduced-intensity (RI)-UCBT (11, 12) represents a promising treatment for advanced hematological malignancies. Wagner *et al.* (12) reported recently the feasibility of RI-UCBT for pediatric patients. However, the feasibility in adult patients remains unclear. We report 30 adult patients with advanced hematological diseases who underwent RI-UCBT after fludarabine, melphalan, and 4 Gy TBI since October 2003 at our institution.

PATIENTS AND METHODS

Study Patients and Donors. Thirty patients with hematological diseases underwent RI-UCBT at Toranomon Hospital between September 2002 and August 2003. All of the patients had hematological disorders that were incurable with conventional treatments and were considered inappropriate for conven-

Received 12/17/03; revised 2/10/04; accepted 2/23/04.

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