

disease. This result strongly suggested that real-time PCR-guided pre-emptive therapy could not completely prevent the occurrence of CMV-GI disease. However, it is possible that the incidence of CMV-GI disease might be lower in patients receiving real-time PCR-guided pre-emptive therapy than in those receiving CMV antigenemia-guided pre-emptive therapy, simply due to the higher sensitivity of real-time PCR.

We conclude that, in the setting of allogeneic HSCT, the occurrence of CMV-GI disease is uniformly observed at a particular frequency even in patients receiving strict CMV surveillance and pre-emptive therapy using the CMV antigenemia assay or PCR, and consider that the latter may be more useful. Consequently, the results of these assays should not be wholly relied on in the diagnosis and prediction of CMV-GI disease. Further modification of CMV antigenemia-guided pre-emptive therapy should focus on using more sensitive PCR methods, and take into consideration individual patient CMV-specific immune reconstitution.

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Reduced intensity conditioning regimens

Feasibility of reduced intensity hematopoietic stem cell transplantation from an HLA-matched unrelated donor

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Summary:

To evaluate the feasibility of reduced intensity stem cell transplantation (RIST) with bone marrow from a matched unrelated donor (MUD), we retrospectively investigated 20 patients with hematological disorders who received RIST in the Tokyo SCT consortium from January 2000 to October 2002. The preparative regimens were fludarabine-based (150–180 mg/m², *n* = 18) or cladribine-based (0.77 mg/kg, *n* = 2). To enhance engraftment, antithymocyte globulin (ATG) and 4 or 8 Gy total body irradiation (TBI) were added to these regimens in nine and 11 patients, respectively. GVHD prophylaxis was cyclosporine with or without methotrexate. In all, 19 achieved primary engraftment. Three developed graft failure (one primary, two secondary), and five died of treatment-related mortality within 100 days of transplant. Seven of the 19 patients who achieved initial engraftment developed grade II–IV acute GVHD, and seven of 13 patients who survived >100 days developed chronic GVHD. At a median follow-up of 5.5 months, estimated 1-year overall survival was 35%. Compared with a TBI-containing regimen, an ATG-containing regimen was associated with a high risk of graft failure (30 vs 0%, *P* = 0.0737). This study supports the feasibility of RIST from MUD; however, procedure-related toxicities remain significant in its application to patients.

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refractory hematological malignancies; however, it has been restricted to young patients without comorbidity. The introduction of reduced intensity stem cell transplantation (RIST) has expanded the treatment option to older, medically infirm patients.^{1,2} Another limitation of the treatment is the stem cell source; HLA-matched related donors are available to only 30% of the patients who require this procedure. An HLA-matched unrelated donor (MUD) is an important alternative donor source.³ The feasibility of RIST from a MUD has not been extensively studied, leaving an optimal conditioning regimen to be determined.

As of October 2002, we had treated 191 patients with hematological diseases or solid tumors with RIST, 20 of whom underwent RIST with unrelated bone marrow (BM). This study retrospectively examined the feasibility of RIST from a MUD.

Patients and methods

Patients and donors

We studied 20 consecutive patients who underwent RIST from a MUD following either antithymocyte globulin (ATG)- or total body irradiation (TBI)-containing conditioning regimens at the Tokyo Stem Cell Transplant Consortium between January 2000 and October 2002. They were eligible for RIST due to age >50 years and/or organ dysfunction. Of the 20 patients, 17 had high-risk hematological malignancies (progressive diseases or those in >2nd remission) (Table 1). The other three patients were classified as having low-risk diseases. All of the patients and donors gave their written informed consent in accordance with the requirements of the Institutional Review Board.

HLA typing and donor matching

An HLA-A, -B, and -DR antigen-matched donor was sought through the Japan Marrow Donation Program (JMDP) as reported previously.⁴ Alleles at HLA-A2, 26, -B39, 61, and 75, which are highly polymorphic in the Japanese population,⁵ and DRB1 were routinely identified

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the treatment of choice for patients with

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Table 1 Patient characteristics

No.	Sex	Age	Primary disease	Disease status at transplant	Karyotype	Previous lines of therapy	ANC × 10 ⁹ /kg	Conditioning regimens	HLA allele mismatch	GVHD prophylaxis
1	F	29	MDS RA	RA	8 tetrasomy	2	2.8	2-CdA/Bu/ATG	1	CsA + PSL
2	F	55	AML M2	Second CR	Normal	1	2.8	2-CdA/Bu/ATG	0	CsA
3	M	52	CML BC	Refractory	t(9;22)	2	1.8	Flu/Bu/ATG	0	CsA
4	F	59	AML M2	First RL	Complex del(5)	1	2.4	Flu/Bu/ATG	0	CsA
5	M	59	ATLL	First RL	—	3	2.5	Flu/CY/ATG	0	CsA/PSL
6	F	71	AML M1	Third CR	Normal	3	0.8	Flu/Bu/ATG	0	CsA
7	M	38	MM	Refractory	Normal	Auto SCT/2	2.5	Flu/Mel/ATG	0	CsA
8	F	35	SAA	—	Normal	3	3.3	Flu/Mel/ATG/TLI4Gy	0	CsA + sMTX
9	M	52	MDS RAEB	Refractory	17(q10)	1	2.5	Flu/BU/ATG	1	CsA
10	M	60	AML	Overt leukemia	Complex	1	2.9	Flu/BU/TBI4Gy	1	CsA + MMF
11	M	65	AML M3	Third CR	t(15;17)	2	2.2	Flu/Mel/TBI8Gy	0	CsA + sMTX
12	F	58	NK lymphoma	First RL	—	2	2.5	Flu/BU/TBI8Gy	0	CsA
13	F	53	MDS	Graft rejection	Normal	Allo SCT/1	3.3	Flu/BU/TBI4Gy	0	CsA
14	M	58	PCL	Refractory	47,XY,+Y	5	1.0	Flu/BU/TBI4Gy	0	CsA
15	F	55	AML M4	Third CR	inv(16)	3	1.8	Flu/BU/TBI4Gy	0	CsA + sMTX
16	M	52	MDS RA	RA	Normal	3	0.8	Flu/BU/TBI4Gy	0	CsA
17	F	50	AML M4	Second CR	inv(16)	4	5.2	Flu/BU/TBI8Gy	0	CsA + sMTX
18	F	50	FL	Refractory	—	2	1.1	Flu/BU/TBI4Gy	0	CsA + sMTX
19	F	52	MDS	Overt leukemia	-7	2	2.2	Flu/BU/TBI4Gy	1	CsA + sMTX
20	F	57	AML M4	Second RL	Normal	4	1.5	Flu/BU/TBI4Gy	0	CsA + sMTX

MDS = myelodysplastic syndrome; RA = refractory anemia; RAEB = RA with excess blasts; ATLL = adult T-cell leukemia lymphoma; AML = acute myeloid leukemia; CML = chronic myeloid leukemia; SAA = severe aplastic anemia; DLBCL = diffuse large B-cell lymphoma; FL = follicular lymphoma; MM = multiple myeloma; PCL = plasma cell leukemia; 2CdA = cladribine; Flu = fludarabine; BU = busulfan; TBI = total body irradiation; TLI = total lymphoid irradiation; ATG = antithymocyte globulin; 2CdA = cladribine; CSP = cyclosporine; PSL = prednisolone; sMTX = short-term methotrexate; MMF = mycophenolate mofetil; CR = complete remission; RL = relapse; Auto = autologous transplantation with high-dose chemotherapy; Allo = allogeneic myeloablative hematopoietic stem cell transplantation; ANC = all nucleated cell.

by high-resolution DNA typing. BM was collected by a standardized technique on the day of infusion.

Preparative regimens

In the National Cancer Center Hospital, the preparative regimens used were cladribine 0.11 mg/kg on days -10 to -4 and busulfan 4 mg/kg on days -6 and -5,⁶ then cladribine was replaced with fludarabine 30 mg/m² on days -8 to -3 as the supply of cladribine was suspended (Table 1). In the Toranomon Hospital, fludarabine was administered in the same schedule and cyclophosphamide was 60 mg/kg on days -3 to -2. Cyclophosphamide was switched to melphalan 140 mg/m² on day -1 because of the number of patients with cardiac dysfunction.

To enhance engraftment, rabbit ATG (thymoglobulin; IMTIX-SANGSTAT, Lyons, France) 2.5 mg/kg/day was administered for 2 (n = 3), 2 (n = 3), and 4 (n = 3) consecutive days, finishing on day -1. ATG was replaced by 4 or 8 Gy fractionated TBI because of the observed high rate of graft failure. TBI was administered on day -1 in two fractions. Case 8 received ATG and total lymph node irradiation, and was classified in the ATG group in this study.

Engraftment and chimerism analysis

Engraftment was defined as a white blood cell (WBC) count of > 1.0 × 10⁹/l or an absolute neutrophil count (ANC) of > 0.5 × 10⁹/l for 2 consecutive days, and a platelet count of > 20 × 10⁹/l for 2 consecutive days without transfusions. G-CSF 300 µg/m²/day was administered intravenously from day 5 till neutrophil engraftment. Secondary graft failure

was defined as peripheral cytopenia and marrow hypoplasia occurring later than day 21 without detection of donor markers by cytogenetic and molecular techniques.

Donor-recipient chimerism was assessed using CD3-positive cells by fluorescent *in situ* hybridization (FISH) in sex-mismatched donor-recipient pairs. In sex-matched pairs, multiplex amplification of short tandem repeats (STR) was used with donor cells detected at a sensitivity of 10%.⁷

Regimen-related toxicity

Regimen-related toxicity (RRT) was defined as all non-hematological organ dysfunctions from day 0 to day 28, and was graded according to the toxicity criteria developed by the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0 (Table 2). Transplant-related mortality (TRM) was nonrelapse mortality.

Supportive cares and management of GVHD

All of the patients were managed in laminar airflow-equipped rooms, and received prophylaxis with trimethoprim/sulfamethoxazole or pentamidine inhalation, ciprofloxacin, fluconazole, and acyclovir.⁸ Neutropenic fever was managed as described by Pizzo.⁹ Cytomegalovirus (CMV) pp65 antigenemia was monitored weekly with initiation of preemptive ganciclovir at positive results.⁸

GVHD prophylaxis was cyclosporin alone (n = 10), or with short-term methotrexate (n = 7), prednisolone 0.5 mg/kg (n = 2), or mycophenolate mofetil (n = 1) (Table 1). In the absence of GVHD, cyclosporin was tapered from day 100 until day 180. GVHD was diagnosed based on clinical

Table 2 Regimen-related toxicity

	Maximal grade ^a			
	1	2	3	4
Mucosa	6	4	3	0
Gut	4	5	2	1
Liver	2	3	3	2
Lung	2	1	3	1
Kidney	4	2	2	0
Heart	0	0	2	0
CNS	0	0	2	0
Bladder	4	1	0	0

^aDetermined by the NCI-CTC version 2.0.

and pathological findings. Acute and chronic GVHD was graded according to the consensus criteria.^{10,11}

End points and statistical analysis

The primary end points were durable engraftment and TRM at day 100. The secondary end points were RRT, incidence of acute and chronic GVHD, event-free survival (EFS), and overall survival (OS). These end points were compared between ATG- and TBI-conditioned groups. EFS was defined as the post-RIST survival duration without disease progression, relapse, graft failure, or death. Probabilities of OS and EFS were calculated as a function of time by the Kaplan–Meier method. Surviving patients were censored on the last day of follow-up.

A univariate analysis using Fisher's exact test and the Mann–Whitney test was performed to compare the clinical characteristics of ATG- and TBI-conditioned groups. A Cox regression analysis was used to determine the effects of several variables on OS. Significant factors were identified based on a forward stepwise procedure. The variables entered in each stepwise analysis were sex, age, disease (acute leukemia vs other, and mature B-cell neoplasms and others), risk of primary disease (high vs low), preparative regimen (TBI-containing vs ATG-containing), HLA-allele matching, time-to-RIST from diagnosis, history of autologous HSCT with high-dose chemotherapy, ABO mismatch, and the number of infused nucleated cells. *P*-values <0.05 were considered significant.

Results

Engraftment

The median number of nucleated cells infused was 2.6×10^8 /recipients' body weight (kg) (range 0.8– 5.2×10^8 /kg). In all, 19 patients (95%) achieved primary neutrophil engraftment, and 12 (60%) reached more than 20×10^9 /l platelets. The median time to recover an ANC of 0.5×10^9 /l was 15 days (10–25), while 20.5 days (11–32) were needed to reach a platelet count above 20×10^9 /l. The median number of transfused red blood cell and platelet products, within 60 days post transplant, was 12 (0–104) and 125 (10–835) units, respectively.

Graft failure

Three patients, all of whom received an ATG-containing preparative regimen, developed graft failure (primary in one and secondary in two). One patient with AML (case 4) who was refractory to conventional chemotherapy did not recover her blood cell counts, and finally died of sustained disease progression on day 48. Two patients (cases 2 and 9) developed secondary graft failure following preemptive use of ganciclovir. These patients did not respond to G-CSF therapy.

Statistical analysis showed a significant association between graft failure and the use of an ATG-containing regimen ($P=0.0491$). Two of the five patients who received an allele-mismatched graft developed graft failure, whereas one of the 15 patients who received a matched graft developed graft failure, and this difference was not statistically significant ($P=0.071$).

Chimerism analysis

Chimerism analysis was available in all but four patients who showed early disease progression (cases 4 and 20) and cytopenia (cases 1 and 2). Of these 16 patients, 15 (94%) achieved full donor T-cell chimerism by day 100. The remaining patient (case 9) had 68% donor chimerism on day 30 and subsequently developed secondary graft failure on day 38.

Toxicity

NCI-CTC grade III–IV toxicity within 28 days post transplantation was observed in seven patients (Table 2). Five patients (25%) died of TRM within 100 days of transplant. The causes of death were pulmonary bleeding due to acute GVHD, interstitial pneumonitis, gastrointestinal bleeding, graft failure, and liver failure.

GVHD

Grade II–IV acute GVHD developed in 7/18 evaluable patients on a median of day 24 (range 19–70; Table 3). Three patients died of acute GVHD. Given the high risk of relapse of the underlying diseases, immunosuppressants were tapered rapidly in two patients (cases 6 and 15) to induce GVHD; case 15 died of acute GVHD and case 6 died of invasive pulmonary aspergillosis following steroid treatment for mild GVHD. Seven of the 11 patients (64%), who survived longer than 100 days, developed chronic GVHD. Chronic GVHD was preceded by acute GVHD in six patients.

Infection

Reactivation of CMV infection was documented in 8/20 (40%), while none of them developed CMV disease. One (5%) developed fungal infection, which led to TRM (Table 3). Case 16 developed hemorrhagic cystitis attributable to adenovirus infection (serotype 11) on day 87, which responded to hydration.

Table 3 Clinical course, graft failure, and GVHD

No.	Graft failure	Neutrophils >0.5 × 10 ⁹ /l	Platelets >20 × 10 ⁹ /l	Chimerism (donor %)	Acute GVHD	Chronic GVHD	CMV reactivation	Best response	Current status (months)	Causes of death
1		14	13	—	0	—	Yes	NC	Dead (2)	Pulmonary bleeding
2	Day 49	14	15	—	0	0	Yes	CR	CCR (41+)	
3		12	16	70	0	Extensive	No	CR	CCR (32+)	
4	Primary	Not evaluable	Not reached	—	—	—	No	PD	DEAD (2)	Disease progression
5		10	Not reached	100	0	—	Yes	CR	Dead (2)	Liver failure
6		12	21	100	II skin/gut	Extensive	Yes	CR	Dead (7)	Invasive aspergillosis
7		13	Not reached	100	0	—	No	PR	Dead (2)	Interstitial pneumonitis
8		19	22	100	II skin	0	Yes	CR	CCR (22+)	
9	Day 38	20	Not reached	68	0	—	No	NC	Dead (2)	Graft failure
10		11	Not reached	100	I skin	Extensive	No	CR	Dead (6)	Pneumonia
11		25	Not reached	100	III skin/gut	Limited	No	CR	Dead (8)	Interstitial pneumonitis
12		11	Not reached	100	I skin	0	No	PR	Dead (5)	Disease progression
13		19	32	100	0	0	Yes	CR	CCR (15+)	
14		23	Not reached	100	III skin	—	Yes	CR	Dead (3)	Acute GVHD
15		98	Not reached	100	II skin/gut	—	Yes	CR	Dead (4)	Acute GVHD
16		12	Not reached	100	I skin	Extensive	Yes	CR	CCR (11+)	
17		15	24	100	II skin	Extensive	Yes	CR	CCR (10+)	
18		16	16	100	III skin/liver/gut	Extensive	Yes	PR	CCR (9+)	
19		20	23	100	0	—	No	NC	Dead (4)	Disease progression
20		17	22	—	—	—	No	PD	Dead (2)	Disease progression

NE = not evaluable; CR = complete remission; PR = partial remission; NC = no change; PD = progressive disease; CCR = continuous CR; — = no data.

Survival

As of August 2003, the median follow-up was 5.5 months (range 2.0–41 months). The estimated 2-year OS and EFS were, respectively, 35.0% (95% confidence interval (CI) 24.7–45.3%) and 30.0% (95% CI 19.8–40.2%) (Figure 1).

Comparison of ATG- and TBI-containing regimens

These two treatment groups are compared in Table 4. While GVHD prophylaxis tended to be more intense in the TBI group than in the ATG group, graft failure developed more frequently in the ATG group than in the TBI group (*P* = 0.074). There was no difference in TRM between TBI and ATG groups.

Discussion

Few studies have been reported on the feasibility of RIST from a MUD.^{12–15} Two studies from Israeli¹³ and Texas groups¹² suggest its feasibility for durable engraftment (25/29 vs 15/16, respectively), while the German study reported that 41/42 patients engrafted followed by 8/41 with secondary graft failure.¹⁴ However, the variety of the conditioning regimens, GVHD prophylaxis, and patient characteristics make the risk factors for graft failure difficult to determine. The present study showed that all the 20 patients engrafted, supporting the feasibility of RIST from MUD. However, two of them developed secondary graft failure, both of whom had received ATG as part of the conditioning regimen. This may suggest a negative effect of ATG on engraftment.

ATG is comprised of polyclonal serum immunoglobulin cultivated in rabbits against T-cell lines. It has observable effects on T cells for up to 4 days after administration.¹⁶ Thus, ATG can deplete both host- and donor-derived T

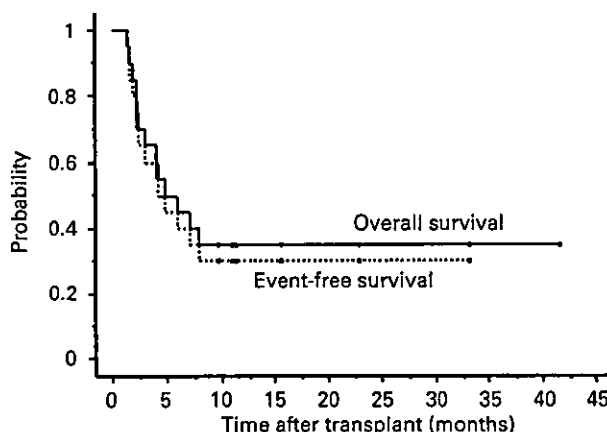


Figure 1 OS and EFS. The estimated 2-year OS (solid lines) and EFS (dotted lines) were, respectively, 35.0% (95% CI 24.7–45.3%) and 30.0% (95% CI 19.8–40.2%).

cells and inhibits GVHD due to its long half-life.¹⁷ In contrast, TBI has no effect on donor-derived T cells. These findings support the contention that the use of ATG in RIST from MUD is associated with a high rate of graft failure, while TBI-containing regimens might enhance engraftment. Since we replaced ATG with TBI, we have not experienced any graft failure. This is consistent with a previous report that TBI-containing RIST from MUD has attained engraftment in patients with aplastic anemia.¹⁸ However, it is to be noted that our purine analog-based regimens using intermediate-dose TBI cause considerable myelosuppression. Since regimen-related toxicities were moderate and acceptable in this study, we consider that our regimens are classified as reduced intensity regimens. Further investigation is warranted to investigate optimal preparative regimens for RIST.

Table 4 Patient characteristics according to conditioning regimen

		ATG group (n = 9)	TBI group (n = 11)	P-value
Secondary graft failure	Yes/no	3/6	0/11	0.073
Grade II-IV acute GVHD	Yes/no	2/7	5/6	0.37
Reactivation of CMV infection	Yes/no	5/4	6/5	> 0.99
Maximal grading of regimen-related toxicity according to NCI-CTC version 2.0	3-4/0-2	4/5	4/7	> 0.99
Transplant-related mortality within 100 days of transplant		4	1	0.13
Estimated 1-year OS (95% confidence interval)		33 (17-49)	36 (21-51)	0.52

Another method to enhance engraftment is use of peripheral blood stem cell (PBSC) transplantation instead of BM.¹⁵ Since PBSC collections contain more CD34-positive and T cells than BM, they may be advantageous in achieving engraftment in RIST from MUD.

GVHD is the most important problem in RIST. In the present study, there were more patients with grade II-IV GVHD in the TBI group (n = 5) than in the ATG group (n = 2) despite additional GVHD prophylaxis with MTX. This may be because ATG reduces the frequency and severity of GVHD by suppressing T cells in the graft. However, day 100 TRM tended to be lower in the TBI group (n = 1) than in the ATG group (n = 4). These results suggest that TBI-containing regimens are safer in RIST from MUD than ATG-containing regimens; however, further studies are required to improve management of GVHD following RIST from MUD.

The incidences of CMV reactivation and disease have been reported to be higher in conventional SCT from MUD (87 and 73%) than conventional SCT from matched siblings (53 and 14%).¹⁹ HSCT from unrelated donors achieves immune reconstitution later than HSCT from related donors, which leads to a higher risk for severe infections.¹⁹ Our study revealed that the incidences of fungal infection and CMV reactivation were, respectively, 15 and 54%, which are comparable to the reports in conventional SCT recipients.^{20,21}

Our results suggest that RIST with BM from MUD is feasible. However, we should comment on some limitations of this study. First, patients who had been enrolled on some pilot studies of RIST were analyzed in this study, and several different preparative regimens and GVHD prophylaxis were utilized. Second, the two comparison groups (ATG vs TBI) had different follow-up periods. This might complicate the interpretation of OS and EFS data when one attempts to compare the two groups. While this study suggests that RIST from MUD is feasible, further studies are required to improve its safety and efficacy. The use of intermediate-dose TBI as an alternative to ATG may enhance engraftment, although the optimal dose of TBI and GVHD prophylaxis regimen remains to be defined.

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The mouse natural killer T cell-associated antigen recognized by U5A2-13 monoclonal antibody is intercellular adhesion molecule-1

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Abstract

Natural Killer T (NKT) cells in mice are generally defined as NK1.1⁺ T cells, although NK1.1 antigen is expressed only in C57BL/6 and related strains. This has precluded investigations of other strains. To find a novel NKT cell surface marker, we generated a monoclonal antibody (mAb), U5A2-13, which recognizes phenotypically and functionally similar populations to NKT cells in naïve mice irrespective of strain. Here, by using a COS-7 expressional cloning system, we molecularly cloned a cDNA encoding a protein reactive with the U5A2-13 mAb and then identified it as intercellular adhesion molecule-1 (ICAM-1). Importantly, the U5A2-13 mAb did not stain hepatic mononuclear cells from ICAM-1 gene disrupted mice. Furthermore, Pepsan method disclosed that the discontinuous epitope for U5A2-13 mAb is composed of three loops located in extracellular domain two of ICAM-1. Overall, U5A2-13, a mAb originally established for mouse NKT cells, recognizes a novel conformational epitope of ICAM-1.

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Keywords: Natural killer T cell; U5A2-13 monoclonal antibody; Intercellular adhesion molecule-1; Molecular cloning

1. Introduction

Natural Killer T (NKT) cells in mice are usually defined as lymphocytes expressing intermediate levels of TCR and NK cell-associated molecules, particularly NK1.1. They predominantly express TCR with invariant V α 14J α 281 and V β 8, 7, or 2, and most of them are phenotypically double negative (CD4⁻CD8⁻) or single positive (CD4⁺CD8⁻) T cells [1–6]. These cells produce large amounts of IFN- γ and IL-4, suggesting that they play an important role in regulating the Th1/Th2 balance [1,2,7–11]. NKT cells are potentiated by α -galactosylceramide (α -GalCer) to produce these cytokines in a CD1d dependent manner [7,8,10,12–17].

NK1.1 antigen is expressed on NK and NKT cells in only C57BL/6 and a few related strains. Therefore, studies of NKT cells in NK1.1-negative strains are quite limited by the lack of markers that can reliably enumerate the population [1,2]. Earlier studies have also shown that NK1.1⁺CD4⁺ T cells lose NK1.1 expression upon in vitro activation [18].

Several surrogate markers such as CD44, Ly6C, Ly49A and DX5 have been postulated. However, these molecules do not accurately encompass NKT cells in C57BL/6 mice and their specificity is difficult to determine [2]. An antibody (Ab) to V α 14⁺ TCR [19] stained less than 20% of V α 14⁺J α 281⁺NK1.1⁺ T cells in thymocytes and is no more used [6]. While tetrameric CD1d molecules loaded with α -GalCer have been used recently to specifically detect V α 14 NKT cells [20,21], NKT cells detected with these tetramers might receive positive signals and become

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activated, making it difficult to ascertain the physiological role of NKT cells in vivo. Furthermore, NKT cells are heterogeneous and, regardless of NK1.1 expression, consist of CD1d dependent and independent subsets [22–27].

To overcome these limitations, we established the monoclonal antibody (mAb), U5A2-13, which selectively identifies populations similar to NK1.1⁺ T cells in both NK1.1-positive and -negative mouse strains [28]. U5A2-13 mAb was originally obtained by immunizing a Fischer rat with tMK-2U lymphoma cells from a BALB/c nude mouse carrying a xenografted human inflammatory breast tumor [29]. We have shown that U5A2-13⁺ T cells use an invariant TCR V β 8, 7, or 2 predominantly and that, similar to NK1.1⁺ T cells, U5A2-13⁺ T cells can produce both IFN- γ and IL-4 upon cross-linking with CD3 [30]. We also demonstrated that hepatic U5A2-13⁺ T cells recognize the NKT cell ligand, α -GalCer, presented by CD1d molecules on dendritic cells [31]. These results indicated that U5A2-13 mAb would be a valuable tool in the study of NKT cells in NK1.1-negative mouse strains.

However, the cell surface molecule recognized by U5A2-13 mAb was unknown. To elucidate the novel cell surface marker constitutively expressed on NKT cells, we molecularly cloned the antigen and identified a new epitope of ICAM-1 on NKT cells. The conformational epitope is composed of three loops located in extra-cellular domain two of ICAM-1.

2. Materials and methods

2.1. Mice

Specific pathogen-free female C57BL/6 wild-type mice (Charles River Japan, Inc., Kanagawa, Japan) and ICAM-1 mutant mice (tm1Bay and tm1Jcgr; Jackson Laboratory, Bar Harbor, ME) maintained in the animal facility of the National Cancer Center Research Institute were studied at 8–12 weeks of age. All animal experiments were conducted in accordance with protocols approved by the institutional review board.

2.2. Antibodies and reagents

FITC-conjugated mAbs specific for mouse CD3 (145-2C11) and ICAM-1 (3E2), PE-conjugated control rat IgG2a (R-35-95), PE-conjugated U5A2-13, PE-conjugated anti-ICAM-1 (3E2), PerCP-conjugated streptavidin, biotinylated anti-CD3 (145-2C11) and anti-mouse Fc γ II/III receptor (2.4G2) were purchased from BD PharMingen (San Diego, CA) for flow cytometry. PE-conjugated anti-NK1.1 mAb, and PE-conjugated anti-rat IgG2a were obtained from Caltag (Burlingame, CA).

Anti-ICAM-1 Ab (goat polyclonal IgG, M-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and peroxidase-conjugated anti-goat IgG was obtained from

Sigma Aldrich (Saint Louis, MO) for Western blotting and immunoprecipitation.

2.3. Cell cultures

The mouse B cell leukemia cell line, BCL1, was cultured in RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) medium supplemented with 10% heat inactivated FCS (JRH Biosciences, Lenexa, KS), 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-ME. COS-7 cells were maintained in DMEM supplemented with 10% heat inactivated FCS.

2.4. Construction of cDNA library

We isolated the gene encoding the antigen recognized by U5A2-13 mAb, using an expressional cloning strategy with COS-7 cells (African green monkey kidney cells expressing the T antigen of simian virus 40) [32]. The cloning source was the murine cell line BCL1, which was cultured in RPMI 1640 with 10% heat inactivated FCS. The cells were harvested for total RNA isolation using guanidinium thiocyanate. Poly(A)⁺ RNA was selected by oligo(dT)-cellulose column chromatography and converted to double-stranded cDNA using oligo(dT) primers containing a *Not* I site. An *Eco*R I adapter was attached and cDNAs greater than 2.0 kb were fractionated by agarose gel electrophoresis, digested with *Not* I and ligated into the *Eco*R I/*Not* I-cleaved pME18S vector (simian virus 40-based mammalian expression vector; GenBank accession no. AB009864) [33]. Transfection into competent DH10B *E. coli* cells yielded approximately 1.0×10^6 independent cDNA clones.

2.5. Screening the cDNA library

DH10B cells carrying the plasmids were fused to COS-7 cells as described by Seed and Aruffo [32]. Three days after fusion, COS-7 cells were detached from the plate by incubation with PBS containing 5 mM EDTA. Cells were collected by centrifugation and resuspended in staining buffer (SB) (PBS containing 5% FCS, 0.5 mM EDTA and 0.05% NaN₃). PE-conjugated U5A2-13 mAb was added and the suspension was incubated at 4 °C for 30 min. The cells were washed three times and resuspended in SB. One percent of the most brightly stained cells were sorted using the fluorescence-activated cell sorter (FACS[®]) Vantage [34]. Plasmid DNA was recovered from the sorted cells and used to transform DH10B *E. coli* cells by electroporation. The transformants were cultured and spheroplast-fused with COS-7 cells for the next round of enrichment. Fourteen colonies were randomly selected after two rounds of enrichment and plasmid DNA was extracted from each colony. Plasmid DNAs purified from these 14 cDNA clones were individually transfected into COS-7 cells using Lipofectamin Plus[®] (Gibco Invitrogen, Carlsbad, CA) and analyzed by flow cytometry. Only one transfectant among the cDNA

clones, BLV-13, bound USA2-13 mAb but not control rat IgG2a.

2.6. DNA sequencing

Both strands of the BLV-13 cDNA clone with an insert of 2.8 kb were sequenced with synthetic oligonucleotide primers and analyzed with an automated fluorescent DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotides and deduced amino acids were analyzed by comparison with the GenBank database.

2.7. Southern blotting

Plasmid DNA (100 ng) purified from each screened cDNA library was digested with *EcoR* I/Not I, resolved by electrophoresis and blotted onto nitrocellulose membranes by alkaline transfer. A hybridization probe of 2.8 kb prepared by PCR using 100 pg BLV-13 cDNA as a template was labeled and detected using the commercially available DIG High Prime DNA Labeling and Detection Starter Kit[®] (Roche, Mannheim, Germany), according to the manufacturer's instructions.

2.8. Western blotting, cell surface biotinylation and immunoprecipitation

The expression of ICAM-1 in COS-7 cells was assessed via immunoblot assay as described [35]. Cell surface proteins of the transfected COS-7 cells were biotinylated using sulfo-succinimidobiotin [36]. After three washes in PBS, cells were suspended at a density of 25×10^6 /ml in 0.9% NaCl with 0.01 M HEPES (pH 8.0) and 0.5 mg/ml of Sulfo-NHS-biotin[®] (Pierce, Rockford, IL) and rocked for 30 min at room temperature. The reaction was terminated by adding 1 M Tris-HCl (pH 7.5) and incubated for an additional 15 min. After centrifugation, cells were disrupted in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1% Triton X-100 in the presence of the protease inhibitors aprotinin (2 μ g/ml), leupeptin (2 μ g/ml) and phenylmethylsulfonyl fluoride (PMSF) (0.57 mM) at 4 °C for 30 min with occasional mixing. After centrifugation, the supernatant served as the cell lysate. The cell lysate was clarified by an incubation with protein G-Sepharose[®] (Pharmacia, Peapack, NJ), immunoprecipitated with USA2-13 mAb and anti-ICAM-1 polyclonal Ab for 2 h, then incubated again with protein G-Sepharose for 1 h. After five washes with lysis buffer, protein was eluted directly by boiling for 5 min in SDS sample buffer. The immunoprecipitates were resolved by 7.5% of SDS-PAGE and electrophoretically transferred onto Immobilon[®] membranes (Millipore, Bedford, MA). Biotinylated protein was detected using avidin and biotinylated horseradish peroxidase macromolecular complex (Vector, Burlingame, CA) and an Enhanced Chemiluminescence (ECL[®]) kit (Amersham, Aylesbury, UK).

2.9. Cell preparations and flow cytometry

Hepatic mononuclear cells (MNCs) were prepared as described [30]. The liver was removed, pressed through 200 μ m gauge stainless steel mesh and suspended in RPMI 1640 containing 5% heat inactivated FCS. After washing with the same medium, the cells were resuspended in 30% Percoll[®] (Amersham Pharmacia Biotech, Uppsala, Sweden) and 65 U/ml heparin and centrifuged at $750 \times g$ for 15 min at room temperature. The cell pellet was collected and erythrocytes in the hepatic MNC suspension were removed using 0.83% ammonium chloride-Tris buffer. The remaining hepatic MNCs were washed in RPMI 1640 and suspended in SB. Except for when anti-rat IgG-PE was used, the cells were incubated beforehand with Fc-block reagent (2.4G2, anti-CD16/32, BD PharMingen). Hepatic MNCs (1×10^6) were incubated with FITC or PE conjugated mAbs for 30 min at 4 °C, then washed and suspended in SB for analysis using a FACS Calibur[®] and Cell Quest[®] software (Becton Dickinson, Mountain View, CA). Except for three-color examinations, cells were gated with propidium iodide (PI). Indirect staining proceeded as described [30].

2.10. Epitope mapping

Peptides were synthesized to include all overlapping linear 30-mers covering the whole mouse ICAM-1 sequence. We also designed peptides in which loops of the five domains were combined in all kinds of orientations, re-creating discontinuous regions of the Ig-like domains. The binding activities of USA2-13 mAb to these peptides were measured by Pepsan Systems (Lelystad, The Netherlands). Briefly, 2230 overlapping 30-mers, off-set one by one, were synthesized and screened using credit-card format mini-PEPSCAN cards (455 peptide format/card) as described previously [37–39]. The binding of antibody to each peptide was tested in a PEPSCAN-based enzyme-linked immuno assay (ELISA). The 455-well credit-card format polyethylene cards, containing the covalently linked peptides, were incubated with antibody USA2-13 (10 μ g/ml, diluted in blocking solution which contains 5% horse-serum (v/v) and 5% ovalbumin (w/v) and 0.05% Tween 20) (4 °C, overnight). After washing the peptides were incubated with anti-rat IgG horseradish peroxidase (dilution 1/1000 in blocking solution) (1 h, 25 °C), and subsequently, after washing the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 ul/ml 3% H₂O₂ were added. After 1 h the color development was measured. The color development of the ELISA was quantified with a CCD-camera and an image processing system. The setup consists of a CCD-camera and a 55 mm lens (Sony CCD Video Camera XC-77RR, Nikon micro-nikkor 55 mm f/2.8 lens), a camera adaptor (Sony Camera adaptor DC-77RR) and the Image Processing Software package Optimas, version 6.5 (Media Cybernetics,

Silver Spring, MD, USA). Optimas runs on a Pentium II computer system.

The identified reacting peptides were colored in a three-dimensional model of whole mouse ICAM-1, 1ic1.pdb, which was derived from the PDB-database [40] and was visualized using the software package Swiss PDB-viewer 3.7b1.

3. Results

3.1. Molecular cloning of a full-length cDNA encoding the antigen recognized by U5A2-13 mAb

We applied an expressional cloning strategy using COS-7 cells to isolate the gene encoding the antigen recognized

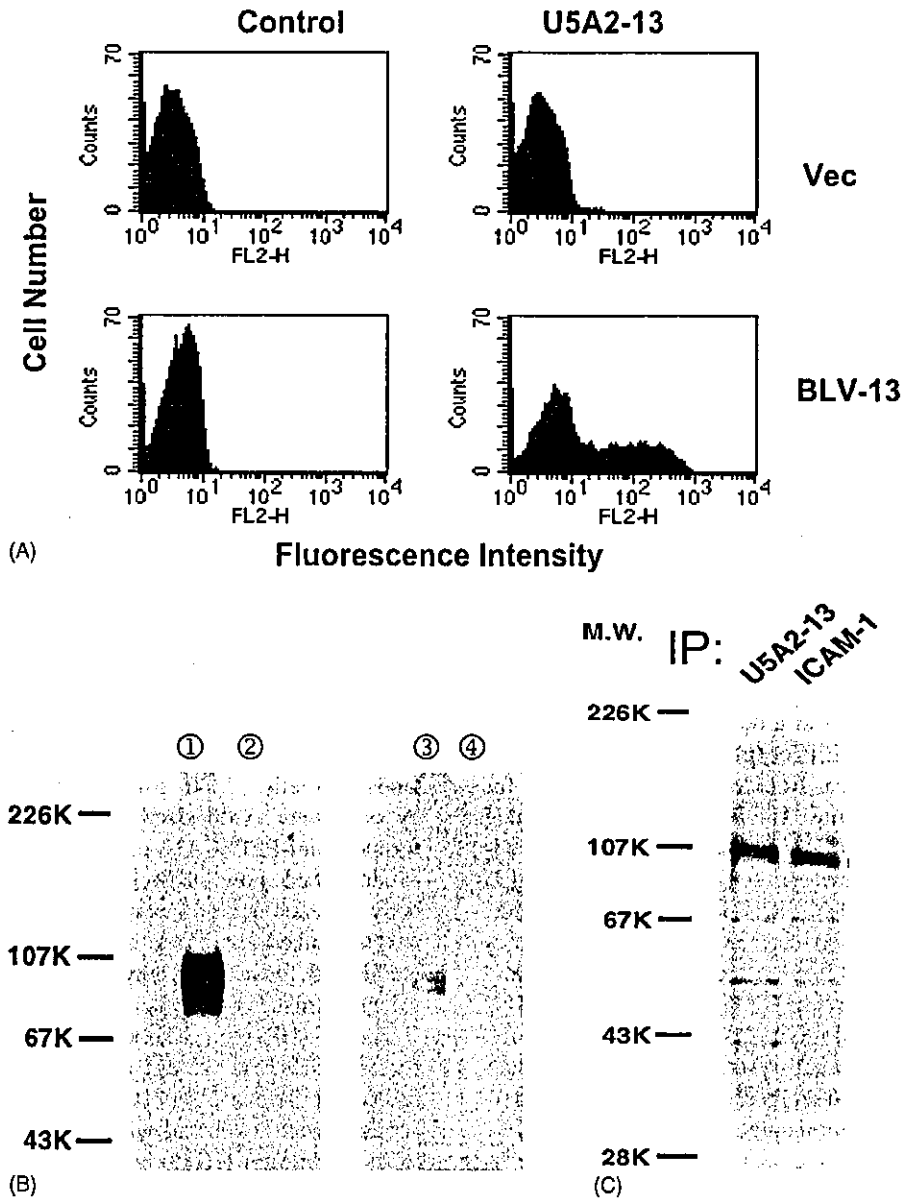


Fig. 1. Surface expression and biochemical analysis of USA2-13-reactive molecules in COS-7 cells transfected with clone BLV-13 cDNA. (A) USA2-13 mAb binds to COS-7 cells transfected with clone BLV-13 cDNA. COS-7 cells were transfected with pME18S vector alone (Vec) or with clone BLV-13 cDNA in pME18S (BLV-13), as indicated, right. Transfectants were stained with either PE-conjugated USA2-13 mAb (USA2-13) or PE-conjugated isotype-matched control Ab (Control) as indicated, above panel. Ab binding was detected by flow cytometry. (B) Western blotting of COS-7 cells transfected with clone BLV-13. Whole lysates of COS-7 cells transfected with clone BLV-13 were Western blotted. Lanes 1 and 2, transfected and control COS-7 cells detected with anti-ICAM-1 Ab, respectively; lanes 3 and 4, transfected and control COS-7 cells stained with anti-ICAM-1 Ab plus its blocking peptides, respectively. (C) Immunoprecipitation analysis of protein encoded by clone BLV-13 cDNA. Cells were labeled with biotin, then proteins were immunoprecipitated with USA2-13 mAb and anti-ICAM-1 Ab. Immunoprecipitates were denatured with SDS sample buffer containing 2-ME and resolved in 7.5% SDS-PAGE gels and blotted. The blot was visualized using avidin-biotinylated horseradish peroxidase macromolecular complex and ECL[®] detection reagents.

by U5A2-13 mAb. A cDNA library was generated from murine BCL1 cells that were positive for U5A2-13 mAb using a pME18S expression vector. Not only NKT cells, but also some murine leukemia cell lines including BCL1, react with U5A2-13 mAb, but BCL1 expresses the most antigen (data not shown). A transfectant of a 2.8 kb cDNA clone, termed BLV-13, that was selected after two cycles of enrichment bound U5A2-13 mAb but not isotype-matched control IgG (Fig. 1A). Before sequencing this cDNA clone, we confirmed its enrichment in the second-round cDNA library by Southern blotting. Sequence analysis demonstrated that BLV-13 cDNA consisted of 2574 bp, encoding a polypeptide with 537 amino acid residues. A GenBank search revealed that the deduced amino acid sequence of BLV-13 was identical to mouse ICAM-1 (GenBank accession no. P13597).

We then Western blotted and immunoprecipitated lysates from BLV-13-transfected COS-7 cells using U5A2-13 and anti-ICAM-1 Abs. A band stained by anti-ICAM-1 Ab in the lysates of BLV-13-transfected COS-7 cells was dimin-

ished by its blocking peptides (Fig. 1B). Immunoprecipitates obtained from BLV-13-transfected COS-7 cells using U5A2-13 and anti-ICAM-1 Abs were detected as a 100 kDa protein band that corresponded to the molecular mass of mouse ICAM-1 (Fig. 1C). To confirm that U5A2-13 mAb reacts with mouse ICAM-1, hepatic mononuclear cells isolated from C57BL/6 mice and ICAM-1 deficient mice were dual-stained with anti-CD3 and one of U5A2-13, anti-ICAM-1 (clone 3E2) or anti-NK1.1 mAbs. We examined two strains of ICAM-1 mutant (exons 4 and 5) mice. U5A2-13 mAb was negative in both strains of ICAM-1 gene targeted mice (Fig. 2). These data demonstrated that our U5A2-13 mAb recognizes ICAM-1.

Furthermore, we sequenced 500 clones obtained from the cDNA library constructed after two cycles of enrichment (data not shown). One-hundred-and-twenty-three out of 500 clones had precisely the same cDNA insert as the 2.8 kb BLV-13 clone, suggesting the absence of an alternative splicing variant other than ICAM-1 mRNA encoding a specific protein recognized by U5A2-13 mAb. No other

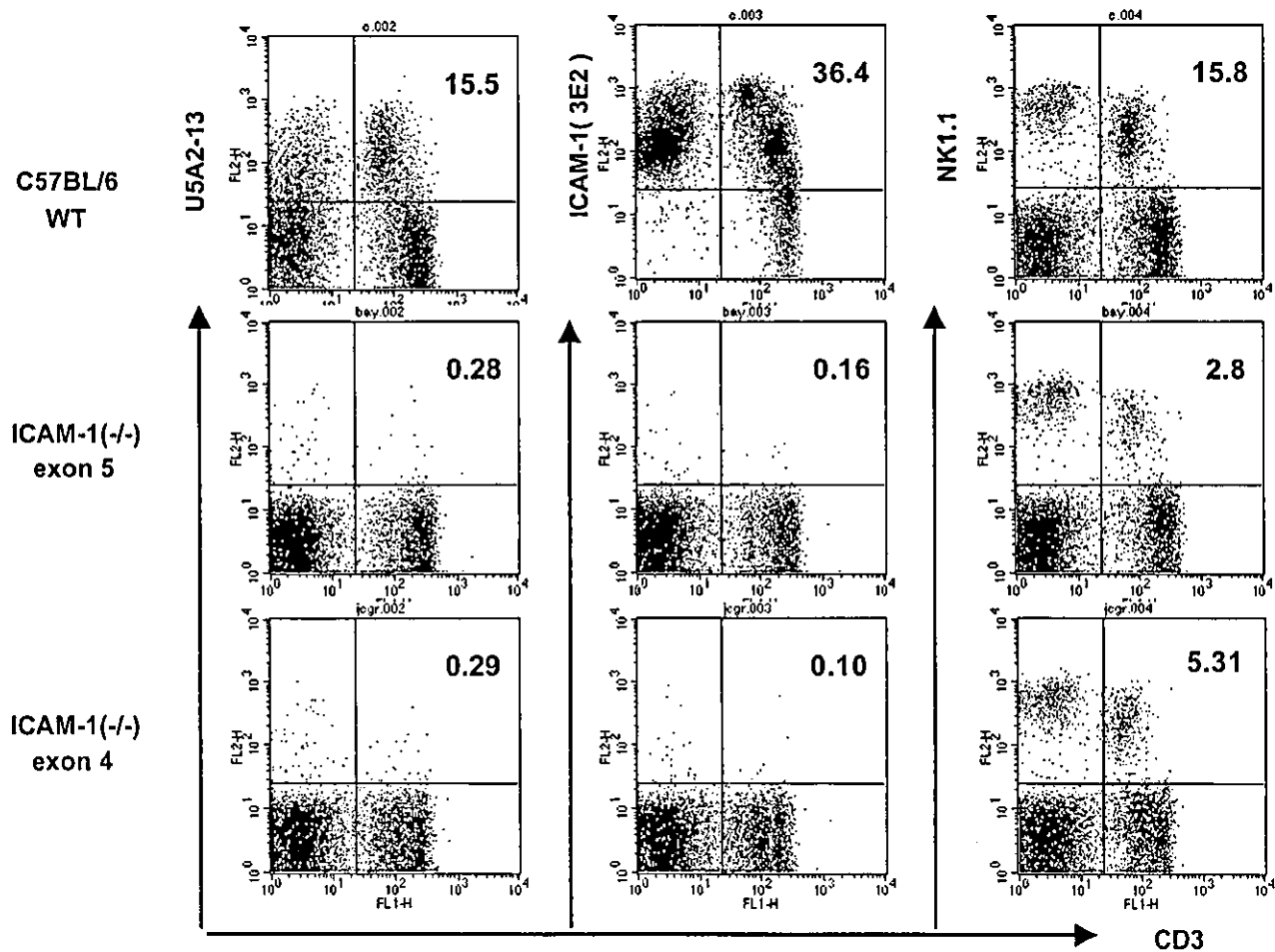


Fig. 2. U5A2-13 mAb did not react with lymphocytes in ICAM-1 deficient mice. Fresh hepatic mononuclear cells were isolated from C57BL/6 and two strains of mice deficient in ICAM-1 (exon 4 mutant and exon 5 mutant) then dual-stained with anti-CD3 mAb and one of U5A2-13 mAb, anti-ICAM-1 (clone 3E2) mAb, or anti-NK1.1 mAb. Data are representative of five individual experiments with similar results.

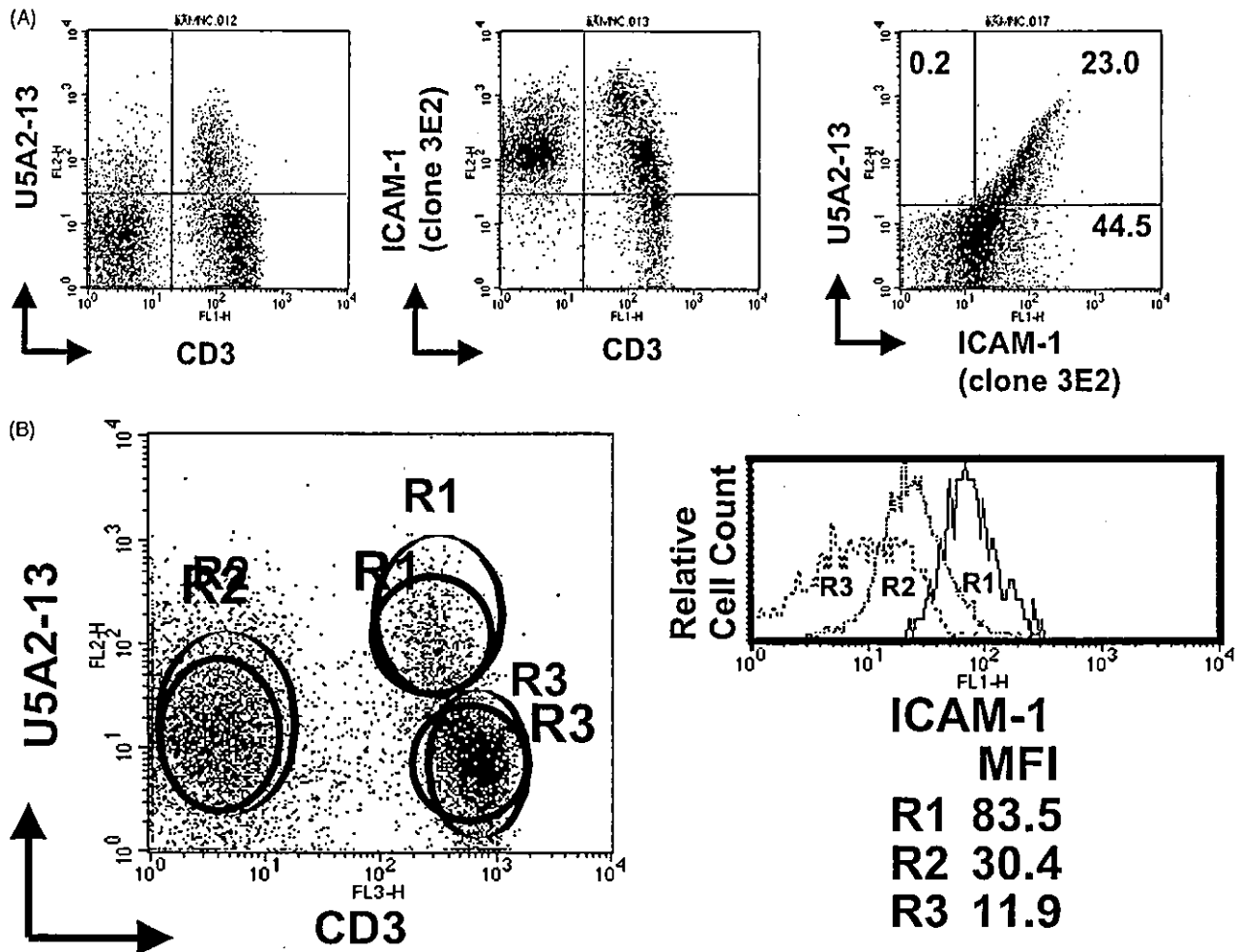


Fig. 3. Flow cytometry revealed that USA2-13⁺ T cells correspond to ICAM-1^{high} T cells. Fresh hepatic mononuclear cells were isolated from C57BL/6 and stained with anti-CD3 mAb, USA2-13 mAb and anti-ICAM-1 (clone 3E2) mAb (A). Mean fluorescence intensity (MFI) of ICAM-1 for each gated region is indicated below the panel (B). Data are representative of three individual experiments with similar results.

genes encoding a cell surface protein were found among the remaining 377 clones.

3.2. NKT cells express an epitope recognized by USA2-13 mAb in extracellular domain two of ICAM-1

The profiles of hepatic mononuclear cells dual-stained with anti-CD3 mAb and one of USA2-13, or anti-ICAM-1 (clone 3E2) mAbs were quite different (Fig. 3A). Three-color flow cytometry demonstrated that the USA2-13 positive population encompasses CD3 positive, ICAM-1 (3E2) bright subsets, corresponding to approximately 35% of the 3E2 positive population (Fig. 3B). Flow cytometry revealed that the mean fluorescence intensity of ICAM-1 is very high not only murine, but also in human TCR V α 24 positive NKT cells (unpublished data).

We examined whether or not the epitopes of the antibodies are different, by performing a competitive inhibition assay. USA2-13 mAb staining was not blocked by a prior

incubation with other anti-ICAM-1 mAbs such as clone 3E2, KAT-1, or YN1/1 (data not shown).

In addition, in order to determine USA2-13 epitope, we generated synthetic peptides that represent amino acid sequences of ICAM-1 and examined their reactivity with USA2-13 mAb by ELISA. Synthetic rod-attached peptides were used to map the antigenic sites on the extracellular domain of the ICAM-1. By means of the Pepscan method, all possible 2230 overlapping 30-mer peptides, composed of two 15-mer parts derived from the primary sequence, were synthesized. USA2-13 mAb was tested against the synthetic minicard-peptides. Peptides were considered to represent antigenic sites if peaks occurred in a set of neighboring peptides and if at least one of the peaks in such a set amounted to more than three times the background. USA2-13 mAb recognized a discontinuous epitope that was made of three parts of extracellular domain two of ICAM-1. The identified sites are: 161–178 region in B–C loop, 135–151 region in C'–E loop, and 188–204 region in F–G loop (Fig. 4).

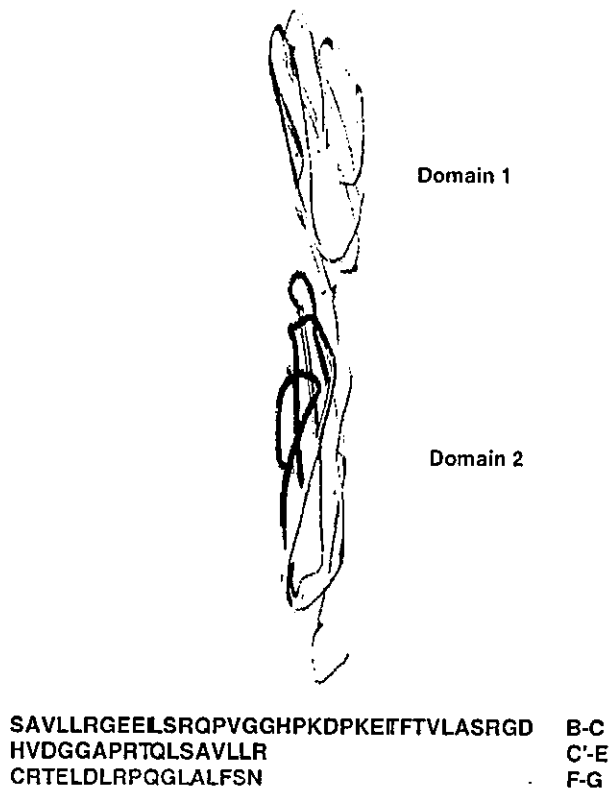


Fig. 4. USA2-13 epitope in a 3D model of human ICAM-1. USA2-13 epitope consists of three loops in domain two of ICAM-1. The three homologous regions are demonstrated in a model of human ICAM-1 (GenBank accession no. MMD8 8008). The core epitope is depicted in red. Two adjacent loops in blue and green are also part of the epitope.

Collectively, these data suggest that NKT cells express a unique epitope of ICAM-1 recognized by USA2-13 mAb.

4. Discussion

Our studies demonstrated that an NKT cell surface antigen recognized by a novel mAb, USA2-13, is encoded by *ICAM-1* gene and that its discontinuous epitope is composed of three loops located in extracellular domain two of ICAM-1. Expression of the classical NK marker, NK1.1 antigen, is confined to C57BL/6 and related strains, which hampers investigation in disease models of NK1.1-negative strains [1,2]. We showed previously that USA2-13⁺ T cells encompass a population functionally similar to NK1.1⁺ T cells in various mouse strains such as C57BL/6, BALB/c, and C3H/He [28,30,31]. Furthermore, staining with USA2-13 mAb yielded higher signal intensity for NKT cells than NK cells in FACS[®] analyses. This is unique compared with antibodies of other NK markers such as NK1.1, IL-2R β , Ly49 or DX5.

Recently the antigen recognized by DX5 has been molecularly cloned, which revealed CD49b (Very Late Antigen-2) [41]. Like USA2-13, a surrogate NK marker, DX5, also corresponds to an adhesion molecule. This is not fortuitous but rather suggests that the importance of adhesion molecules

cannot be over-emphasized in NK and NKT cell function. NKT cells are considered to activate NK cells rapidly in the innate immune system [9]. NK cells express large amount of LFA-1 [42]. Our study showed that NKT cells express ICAM-1 most. This constitutes a reasonable evidence for efficient cross-talk between these cells.

The staining profiles of hepatic MNCs with USA2-13 mAb and with other conventional anti-ICAM-1 mAbs (such as 3E2, YNI/1 or KAT-1) were quite different (Fig. 3A). USA2-13 epitope resides in domain two. The epitopes of the other anti-ICAM-1 antibodies, on the other hand, are located in domain one, which is the site responsible for binding with its ligand LFA-1. Therefore, in competitive inhibition assay, staining with USA2-13 mAb was not blocked by these antibodies (unpublished observation). We cannot exclude a possibility that, because of its affinity for ICAM-1, USA2-13 mAb recognizes only the ICAM-1^{high} population when ICAM-1 is present in substantial amounts on the cell surface. Another possibility is that freshly isolated naïve NKT cells express a unique epitope in domain two of ICAM-1 in such a manner that it can efficiently react with USA2-13 mAb. The ICAM-1 epitope recognized by USA2-13 mAb may depend on glycosylation selective to NK cells. Accumulating evidence shows that ICAM-1 forms homodimers on the cell surface that are more efficient than monomers in binding to its ligand, LFA-1 [43–46]. In freshly harvested mononuclear cells, staining with USA2-13 mAb is mostly confined to NKT cells but not to conventional T and B cells. We also revealed that, upon activation by a mitogen, not only NKT cells but also T and B cells start to react with USA2-13 mAb [47]. Taken together, these findings may indicate that homodimers of ICAM-1 on the surface of activated ICAM-1^{high} cells form an epitope for USA2-13 mAb. ICAM-1 per se is widely expressed on lymphocytes but it is not until they get fully activated and become ICAM-1^{high} when they begin to show USA2-13 epitope otherwise concealed. Similarly, NK1.1 antigen is also induced on CD8⁺ T cells upon activation [48].

It is of interest that this USA2-13 epitope on NKT cells is related to their recognition and functions. In vivo administration of USA2-13 mAb not only modulates cytokines production by NKT cells upon stimulation with α -GalCer but also increases tumor metastases in a B16 melanoma model (manuscript in preparation). We conclude that NKT cells express a unique USA2-13 epitope of ICAM-1.

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Infections post transplant

A nationwide survey of deep fungal infections and fungal prophylaxis after hematopoietic stem cell transplantation in Japan

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Summary:

We conducted a nationwide survey to define incidence of deep fungal infections and fungal prophylaxis practices after HSCT. In all, 63 institutions responded. Total number of in-patient transplantations was 935: 367 autologous, 414 allogeneic myeloablative, and 154 allogeneic reduced-intensity (RIST) ($n=154$). Number of patients who were cared for in a clean room at transplant was 261 (71%) in autologous, 409 (99%) in conventional and 93 (66%) in RIST, respectively. All patients received prophylactic antifungal agents; 89% fluconazole. Number of patients who received the dosage recommended in the CDC guidelines (400 mg/day) was 135 (42%) in conventional transplant and 34 (30%) in RIST ($P=0.037$). Number of patients who received fluconazole until engraftment and beyond day 75 in conventional transplant vs RIST was, respectively, 324 (100%) vs 109 (97%), and 39 (12%) vs 18 (16%), with no significant difference between the two groups. A total of 37 patients (4.0%) were diagnosed with deep fungal infections; autologous transplantation (0.03%), conventional transplantation (6.0%) and RIST (7.1%). Wide variations in antifungal prophylaxis practice according to the type of transplant and the institutions, and deep fungal infection remain significant problems in RIST.

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Fungal infection is a common complication after hematopoietic stem cell transplantation (HSCT), and the primary causative organisms are *Candida* and *Aspergillus*, with significant mortalities even if properly treated with antifungal agents.¹ Therefore, antifungal prophylaxis has been emphasized following HSCT.² In 2000, the Centers for Disease Control and Prevention (CDC) in the United States issued guidelines for the prevention of fungal infections in the setting of allogeneic HSCT and some cases of autologous transplantation,³ and these are considered a gold standard in many countries throughout the world, including Japan. To prevent *Candida* infections, the CDC guidelines recommend the use of fluconazole (400 mg/day) until engraftment,³ based on the results of two randomized controlled studies published in 1992 and 1995.^{4,5} However, since then, the circumstances surrounding HSCT have been changing rapidly. The use of fluconazole has been questioned, since it is ineffective against *Aspergillus* species. Moreover, the rationale for selecting the recommended dose of 400 mg/day fluconazole and the optimal duration of prophylaxis need to be clarified.⁶ A major concern regarding emerging fluconazole-resistant *Candida* species needs to be critically evaluated.^{7,8}

Aspergillus is the most common pathogen in fungal infections in the course of transplantation or treatment for leukemia.⁹ The importance of hospital environment control has been emphasized for effective prevention. The outbreak

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of nosocomial infections due to the renovation of transplant buildings^{10,11} and via the water system in transplant wards has been reported.¹² The CDC guidelines recommend the use of air conditioning systems/tools.³ However, the guidelines may have become outdated due to changing circumstances. First, the timing of the development of aspergillosis has changed. Most *Aspergillus* infections occur late beyond 100 days after transplantation.¹³ This trend may be enhanced by the wider application of reduced-intensity stem cell transplantation (RIST) on an outpatient basis.¹⁴ Second, newly developed antifungal agents have been shown to be effective in the prevention of *Aspergillus* infection.^{15,16} Moreover, a recent drastic change is that RIST has been extended to unrelated transplantation,¹⁷ umbilical cord blood transplantation¹⁸ and elderly patients.¹⁹ RIST has the advantage of a shorter period of neutropenia, and it is likely that the incidence of *Candida* infection is reduced. On the other hand, the incidence of steroid-therapy requiring GVHD remains the same as in conventional transplantations, and the intensity of immune suppression is similar.²⁰ It is expected that late-occurring fungal infections by *Aspergillus*, etc may become a significant problem after RIST.²¹⁻²³

Thus, a re-evaluation and update of the guidelines appears to be necessary, particularly in the area of RIST. There have been only a few studies on fungal infections after RIST.²¹⁻²³ The objective of this study was to survey antifungal prophylaxis in Japanese transplantation practice, and to compare the findings in RIST, conventional allogeneic transplantation (CST), and autologous transplantation.

Patients and methods

Data collection

Of the 418 Japanese medical institutions in which HSCT is performed, 122 institutions agreed to participate in this survey. Each of these institutions received a questionnaire that included the following items: the number of transplantations performed in 2001, the type of the patient's disease and the type of transplantation, use of a clean room or designated room for transplantation, practice regarding prophylactic use of antifungal agents, and occurrence of systemic fungal infections. For cases that developed deep fungal infections, age, sex, date of onset, diagnostic approaches, infected organs, pathogens, neutrophil count at onset, coexistence of GVHD, use of immunosuppressants, prophylactic use of antifungal agents, and patient outcome were also recorded.

We used the EORTC/NIH-MSG criteria for the diagnosis of deep fungal infections.²⁴ We defined both proven and probable infectious cases as deep fungal infection.

Antifungal prophylaxis measures

We defined the nine measures listed below as measures for preventing fungal infection. All of the methods were evaluated as A or B based on the evidence level in the

CDC guidelines, as previously reported.²⁵ The guidelines use a combination of category (A-D) and the recommendation (I-III). We used two recommendation levels, A and B, which indicated strong or moderate evidence supported by well-established clinical trials (I or II) or respected authorities (III).

(a) Yeast infection following allogeneic HSCT

- (a1) Medical staff in contact with HSCT recipients should follow appropriate hand-washing practices to safeguard patients from exposure (Evidence level A III).
- (a2) Fluconazole (400 mg/day, orally or intravenously) should be administered from the day of transplant until engraftment (Evidence level A II).

(b) Mold infections following allo-SCT

- (b3) HSCT recipients who remain immunocompromised should avoid hospital construction or renovation areas (Evidence level A III).
- (b4) Use of high efficiency particulate air (HEPA) filtration (Evidence level B III).
- (b5) Air should flow from patient rooms to corridors (Evidence level B III).
- (b6) Correctly sealed rooms, including correctly sealed windows and electrical outlets (Evidence level B III).
- (b7) High rates of room air exchange (ie, >12 air changes/h) (Evidence level B III).
- (b8) Barriers between patient care areas and renovation or construction areas to prevent dust from entering patient care areas. The barriers must be impermeable to *Aspergillus* species.

(c) Autologous HSCT

- (c9) Autologous HSCT recipients generally are at lower risk of invasive fungal infection than allogeneic HSCT recipients. Autologous HSCT recipients do not require routine intense anti-yeast prophylaxis. Nevertheless, some researchers recommend the use of an anti-yeast prophylaxis in patients who have underlying hematologic malignancies, and who have or will have prolonged neutropenia and mucosal damage from intense conditioning regimens or graft manipulation, or who have recently received purine analogues (Evidence level B III). Regarding mold infections, no guideline has been reported for autologous HSCT.

Prophylaxis for deep fungal infections in Japan

In Japan, the antifungal agents approved for the treatment of deep fungal infections in 2001 included amphotericin B, fluconazole, itraconazole, miconazole and terbinafine. With the exception of itraconazole, they can be administered either orally or intravenously. The only available formula of itraconazole was a capsule, and in this form, absorption through the gastrointestinal tract is inconsistent.²⁶ Itraconazole oral solution, voriconazole, liposomal amphotericin B and echinocandin have not yet been approved.

Governmental approval has not yet been granted for the prophylactic use of any antifungal agents, although this is widely applied as a practice in most medical institutions.²⁷ Fluconazole, up to 400 mg, is approved only for the treatment of fungal infection.

Use of a clean room within a transplant ward is reimbursed by insurance. We considered that prophylactic measures (b3)–(b8) were satisfied if HSCT was performed in a clean room. A clean room was defined as an isolated room equipped with HEPA filtration and air flow toward an exit of the room with sealed windows.

End points and statistical methods

The objective of this study was to conduct a survey of antifungal prophylaxis in HSCT. The levels of compliance with the established prophylactic measures in RIST and CST were compared. The second objective was to compare the CST, RIST and auto-SCT groups with respect to the incidence and characteristics of fungal infections.

We examined (a2) and (b3)–(b8) of the nine items listed above. Item (a1) was excluded from the survey because of inconsistency of proper evaluation in a retrospective survey. We considered that items (b3)–(b8) were satisfied when a clean room was used. We aimed to evaluate differences in fungal prophylaxis between conventional and reduced-intensity transplants. We did not collect detailed information on transplantation procedures such as stem cell sources and drugs used in the preparative regimens. A univariate analysis using Fisher's exact test and the Mann-Whitney *U* test was performed to compare the differences in prophylactic measures between RIST and CST. Values of $P < 0.05$ were considered significant.

Results

Patients background

We received questionnaires from 63 medical institutions, representing 935 transplantations. In Japan, a total of 1964 transplantations were performed in 2001,²⁸ and approximately half of the patients were surveyed in this study. The median number of transplantations per institute/year was 10 (range, 1–108). The types of transplantation were autologous HSCT (367, 40%), CST (414, 45%), and RIST (154, 15%). All of the RIST recipients received purine-analog-based preparative regimens with or without low-dose TBI.

Patients' diseases included malignant lymphoma (191 cases, 21%), acute myelocytic leukemia (147, 16%), acute lymphocytic leukemia (107, 11%), multiple myeloma (80, 9%), chronic myelocytic leukemia (74, 8%), myelodysplastic syndrome (51, 5%), solid tumors (50, 5%), aplastic anemia (16, 2%), and others (219, 23%).

All patients were hospitalized during transplantation. The number of autologous HSCT, CST and RIST recipients who were in a clean room at the time of transplantation was 261/367 (71%), 409/414 (99%) and 93/154 (66%), respectively.

Prophylactic use of antifungal agents

All patients including autologous HSCT cases received an antifungal agent as a prophylaxis, and azole antifungal agents were administered in 742 patients (79%). The most commonly used azole antifungal agent was fluconazole (89%). Figure 1 shows the types of agents used in CST, RIST and autologous HSCT.

Fluconazole was administered to 324 (78%) CST and 112 (73%) RIST recipients. The numbers of patients who received fluconazole 400, 200 and 100 mg/day were 135 (42%), 169 (52%) and 20 (6%), respectively. Those patients who were given fluconazole 400, 200 and 100 mg/day were 34 (30%), 70 (63%) and 8 (7%), respectively. Significantly larger doses of fluconazole were used in CST than in RIST ($P = 0.037$).

The numbers of patients who received fluconazole until engraftment and beyond day 75 in the CST and RIST groups were, respectively, 324 (100%) and 39 (12%), and 109 (97%) and 18 (16%). Duration of fluconazole use was not significantly different between CST and RIST.

Incidence and clinical characteristics of deep fungal infections

Of the 935 transplant cases, 37 (4.0%) were diagnosed with deep fungal infections (13 proven and 24 probable cases); 0.03% (1/367) of autologous HSCT, 6.0% (25/414) of CST, and 7.1% (11/154) of RIST. The causative organisms included *Candida* ($n = 9$), *Aspergillus* ($n = 16$), *Mucor* ($n = 1$), *Fusarium* ($n = 1$) and unknown ($n = 10$). The median onset date from the time of transplant was 85 days (range, 1–392 days): 92 days in CST and 117 days in RIST, with nine cases developing within 30 days of transplant (one case in autologous HSCT, seven in CST and one in RIST). Three cases of 37 deep fungal infections had a previous history of fungal infection. All of them received fluconazole and AMPH-B intravenously as a prophylaxis.

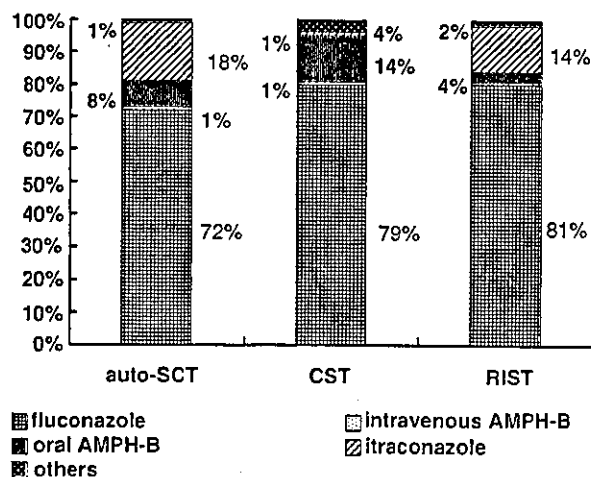


Figure 1 Types of prophylactic antifungal agents used in each type of HSCT. Azole agents were the most-administered drugs for prophylactic use in HSCT patients.

The causative organisms for the nine cases of candidiasis included *C. albicans* (n=4), *C. glabrata* (n=2), *C. tropicalis* (n=1), *C. inconspicua* (n=1), and a combination of *C. lusitaniae* and *C. guilliermondii* (n=1). The infected organs included blood (fungemia, six cases), the respiratory system (n=2) and the liver and spleen (n=1). Among four patients who were infected with *C. albicans*, two had not received any antifungal agents including fluconazole as a prophylaxis, while the remaining two cases had been on fluconazole prophylaxis and were considered to be prophylaxis failure. Among these four patients, three started or continued to receive fluconazole following infection, and three patients subsequently died.

The causative organisms for the 16 cases of aspergillosis included *Aspergillus* spp. (n=14), *A. fumigatus* (n=1) and *A. terreus* (n=1). Six and 10 of these patients received RIST and CST, respectively. In all, 14 and two patients had received prophylactic fluconazole and itraconazole, respectively. GVHD requiring corticosteroid therapy was documented at the onset of the disease in 13 of the 16 cases. The initially infected organ was the respiratory system in all cases, with a median onset of invasive aspergillosis of 85 days (range, 9–392). Four of the 16 patients were neutropenic at the diagnosis of aspergillosis. Treatment for invasive aspergillosis included intravenous AMPH-B (13 cases), oral itraconazole (two cases), and fluconazole (one case). Eight patients subsequently died without resolution of invasive aspergillosis.

Comparison of CST and RIST recipients

Table 1 shows the characteristics of fungal infections developed in CST and RIST recipients. There was no significant difference between the two groups with respect to the incidence, date of onset or death rate.

Discussion

This study highlights the current problems in antifungal prophylaxis. In Japan, a total of 1964 transplantations were performed in 2001,²⁸ and about half of these were surveyed in this study. We believe that sufficient data were collected to characterize prophylaxis practice and fungal infection in this country, while it is limited by bias as a retrospective design, and we should be careful in interpreting the results of this study.

With improvements in prophylactic measures, the incidence of early mold infections has decreased,^{14,29,30}

and most infections occur late in association with GVHD.¹³ The need for a HEPA filter or laminar air flow (LAF) may be questioned in RIST. All transplant wards in Japan, but not all nontransplant wards, are equipped with these systems. Although 99% of CST was performed in transplant wards, this percentage decreased to 71% in autografts and 66% in RIST. The potential economic benefits of RIST may be related to an increase in RIST performed in nontransplant wards without expensive air filtration systems.

In autologous HSCT, the prophylactic use of antifungal agents is recommended only for high-risk patients³ based on several clinical studies.^{4,31} However, this study demonstrates that all of the Japanese autologous HSCT recipients received some prophylactic antifungal agents. This is an overuse of antifungal agents because the overall risk of fungal infection in autologous transplants is lower than allogeneic transplant. There are several possible explanations for this overuse. Since the definition of 'low-risk patients' in the CDC guidelines is unclear, physicians take a more conservative way to reduce the risk of fungal infection. It has been suggested that long-term use of azole antifungal agents may cause resistance.³² Additionally, azole antifungal agents are expensive, and long-term use is not economical.³³ On the other hand, for the prophylactic use of antifungal agents in allogeneic transplant, the CDC recommends prophylactic fluconazole 400 mg/day during the neutropenic period following transplantation. However, the rationale for the selection of fluconazole from among many other available antifungal agents, its dose setting and prophylaxis period remain unclear. Its usefulness in RIST recipients has not been established.

After the CDC issued its guidelines in 2000, various drugs, including voriconazole, itraconazole (oral solution and intravenous formulation), and echinocandin, have been developed and are now commercially available. Moreover, *Aspergillus* is not susceptible to fluconazole, and recent comparative studies suggest that itraconazole is more effective than fluconazole in the prevention of *Aspergillus* infection.^{15,34} Hence, the recommendation of fluconazole over any other agent may no longer be defensible. Nevertheless, this study clearly documented that fluconazole was used in 75% of allogeneic transplant in Japan. This bias is likely due to the CDC recommendation and to the fact that an alternative antifungal agent is not commercially available in Japan. As of June 2003, the only available itraconazole formula in Japan is a capsule, which carries an obvious risk of inadequate

Table 1 Comparison on clinical features of deep fungal infection between CST and RIST

	CST (n=414)	RIST (n=154)
Number of deep fungal infection	26 (6.3%)	11 (7.1%)
Median onset (days after transplant) (range)	77 (1–392)	117 (28–182)
Presence of GVHD at the onset of fungal infection	Present/absent 12/14	9/2
Presence of neutropenia at the onset of fungal infection*	Present/absent 8/18	1/10
Use of corticosteroid at the onset of fungal infection	Yes/no 15/11	7/4
Causative organisms	<i>Aspergillus</i> / <i>Candida</i> /Others 17/7/2	9/1/1
Mortality	81%	82%

*Neutropenia was defined as neutrophils below 0.5 × 10⁹/l.