

Table 1. Patient-, disease-, and transplantation-related characteristics

Variable	No. (%) [*]
Patient characteristics	
Younger than 40 y	158 (68)
40 y or older	75 (32)
Male sex	150 (64)
Disease characteristics at diagnosis	
Histology	
Indolent	38 (16)
Follicular	37 (16)
MALT	1 (0)
Aggressive	111 (48)
Diffuse large B cell	44 (19)
Peripheral T cell, unspecified	22 (9)
Extranodal NK/T cell, nasal type	19 (8)
Anaplastic large cell	7 (3)
Mantle cell	5 (2)
Others	14 (6)
Lymphoblastic	84 (36)
Precursor B cell	7 (3)
Precursor T cell	77 (33)
Stage I	9 (4)
Stage II	25 (11)
Stage III	30 (13)
Stage IV	150 (64)
No data	19 (8)
Disease characteristics at transplantation	
Response to chemotherapy†	
Sensitive	128 (55)
Complete remission‡	90 (39)
Partial remission	38 (16)
Resistant	104 (45)
Primary refractory disease	41 (18)
Refractory relapse	63 (27)
No. of prior chemotherapy regimens†	3 (0-11)
Fewer than 4 regimens	143 (61)
At least 4 regimens	90 (39)
Prior autograft	40 (17)
Prior radiotherapy	81 (35)
Transplantation characteristics	
Year of transplantation	
1990-1995	46 (20)
1996-2001	187 (80)
No. of patients receiving a transplant per hospital	
Fewer than 9 patients	146 (63)
At least 9 patients	87 (37)
Donor	
HLA-matched related	154 (66)
HLA-1 antigen-mismatched related	19 (8)
HLA-matched unrelated	43 (19)
HLA-1 antigen-mismatched unrelated	17 (7)
Donor-recipient sex match	
Male-male	80 (34)
Male-female	66 (28)
Female-male	33 (14)
Female-female	46 (20)
Donor-recipient CMV status§	
+/+	131 (57)
-/+	14 (6)
+/-	14 (6)
-/-	11 (5)
Source of stem cells	
Bone marrow	159 (68)
Peripheral blood cells	70 (30)
Bone marrow + peripheral blood cells	2 (1)
Cord blood	2 (1)

Table 1. Continued

Variable	No. (%) [*]
Conditioning regimen	
TBI-containing	193 (83)
Non-TBI	40 (17)
GVHD prophylaxis	
Cyclosporin + methotrexate	204 (88)
Tacrolimus + methotrexate	22 (9)
Others	7 (3)

The study included 233 patients. The median age was 31 years (range, 15-59 years). Age was a continuous variable.

MALT indicates extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; NK, natural killer; HLA, human leukocyte antigen; CMV, cytomegalovirus; TBI, total body irradiation; GVHD, graft-versus-host disease.

^{*}Categoric variable.

†One patient with mediastinal B-LBL did not receive prior chemotherapy for an unknown reason but did receive prior radiotherapy.

‡Includes 2 patients in complete remission, unconfirmed.

§Sixty-three pairs were not evaluated for CMV status.

GVHD occurred in 33 (19%) and 16 (27%). In allo-HSCT from HLA-matched ($n = 197$) and mismatched ($n = 36$) donors, grade II to IV acute GVHD occurred, respectively, in 76 (39%) and 15 (42%), grade III to IV acute GVHD occurred in 30 (15%) and 7 (19%), chronic GVHD occurred in 65 (33%) and 14 (39%), and chronic extensive GVHD occurred in 41 (21%) and 7 (19%). The distribution pattern of the incidences of acute and chronic GVHD by background factors was analyzed by using a chi-square test. Although none of the factors correlated with acute GVHD, the incidence of chronic GVHD was higher in patients who had GVHD prophylaxis with tacrolimus plus methotrexate than in those with cyclosporin plus methotrexate ($P = .015$, chi-square test; $P = 0.023$, Fisher exact test).

Disease response

Of the 143 patients who had measurable disease at allo-HSCT, 89 (62%) achieved CR, 7 (5%) PR, 6 (4%) stable disease (SD), and 12 (8%) PD, whereas 29 (20%) were not evaluable because of early death. Of the 90 patients who were in CR at transplantation, 80 (89%) maintained CR, 4 (4%) showed PD, and 6 (7%) were not evaluable because of early death. Thirty-five patients died before the first response evaluation, with a median survival of 29 days (range, 0-72 days) after allo-HSCT. In the 27 patients with indolent lymphoma who had measurable disease at allo-HSCT, 22 (81%) achieved CR or PR. In the 72 patients with aggressive lymphoma who had measurable disease at allo-HSCT, 49 (68%) achieved CR or PR. In the 41 patients with lymphoblastic lymphoma who had measurable disease at allo-HSCT, 26 (63%) achieved CR.

TRM, disease relapse, and progression

Ninety-eight patients (42%) died of TRM, and its cumulative incidence is shown in Figure 1. Of the 98 patients who died of therapy-related complications, 60 (61%) died within day 100 of transplantation and 38 (39%) died thereafter. The major causes of TRM included GVHD ($n = 11$), infection ($n = 29$), interstitial pneumonitis ($n = 16$), venoocclusive disease of the liver ($n = 11$), thrombotic microangiopathy ($n = 8$), heart failure ($n = 7$), hemorrhage ($n = 4$), renal failure ($n = 3$), and others ($n = 9$), as shown in Table 2. The causes of infection-related mortality ($n = 29$) were bacterial ($n = 13$), fungal ($n = 11$), or viral ($n = 5$). Seventeen (59%) of 29 patients died of infections within 100 days of allo-HSCT, 7 (24%) from 101 days to 1 year and 5 (17%) thereafter. Fourteen patients died of TRM before engraftment. Of the 98 patients who died of TRM, 67 (68%) had GVHD, and 11 of

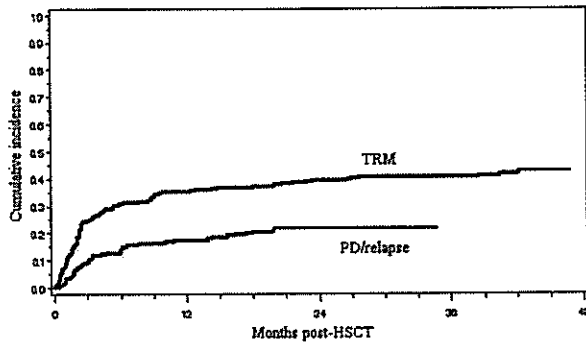


Figure 1. Cumulative incidences of treatment-related mortality (TRM) and disease relapse/progression (PD/relapse).

these died of GVHD (6 acute, 5 chronic) itself. The 14 factors shown in Table 3 were assessed with regard to their relation to TRM. A univariate analysis revealed that 6 factors, including older patient age, chemoresistant disease, prior autograft, prior radiotherapy, aggressive lymphoma other than PTCL, and chronic GVHD, were associated with a significantly increased risk of TRM. In a multivariate analysis using a logistic model, chemoresistant disease, prior autograft, and chronic GVHD remained significant.

The cumulative incidence of relapse and PD is shown in Figure 1. Relapse or progression of lymphoma after allo-HSCT was observed in 49 patients (21%; 5 indolent, 19 aggressive, 25 LBL), and 32 (14%; 3 indolent, 13 aggressive, and 16 LBL) died of PD. Of the 105 patients with chemoresistant disease before allo-HSCT, 61 (58%) died of treatment-related complications, 19 (18%) died of PD, and 25 (24%) are alive with a median follow-up of 20.9 months (range, 1.8-136.0 months). Of the 128 patients with chemosensitive disease before allo-HSCT, 37 (29%) died of treatment-related complications, 12 (9%) died of PD, and 79 (62%) are alive with a median follow-up of 35.2 months (range, 4.4-140.2 months). Eight (16%) of the 49 patients who showed PD died of treatment-related complications such as infection (n = 4), interstitial pneumonitis (n = 3), and GVHD (n = 1). Only 6 of the 70 patients who had passed 2 years after transplantation developed relapse thereafter.

Donor lymphocyte infusion

Donor lymphocyte infusions (DLIs) were given after the withdrawal of immunosuppressive therapy to those who relapsed or showed evidence of disease progression or persistent disease without any sign of GVHD. A total of 7 patients, including 5 with

T-LBL, received DLI after allo-HSCT from an HLA-matched related donor (n = 6) or a -matched unrelated donor (n = 1). Two patients who received DLI from an HLA-matched related donor developed grade II acute GVHD, which subsequently extended to extensive chronic GVHD; one of them with T-LBL died without a response, whereas the other with T-cell lymphoma is still alive without disease progression 3.8 years after allo-HSCT. Five patients did not develop GVHD following DLI; 3 patients subsequently died of disease progression, but 2 patients with T-LBL are still alive without disease progression at 361 and 783 days after allo-HSCT.

OS and PFS

One hundred four (45%) of the 233 patients are currently alive with a median follow-up of 31 months (range, 1.8-138 months). The OS and PFS are, respectively, 45% and 40% at 2 years, and 39% and 36% at 5 years after allo-HSCT (Figure 2). Median OS and PFS are, respectively, 15.6 months (95% confidence interval, 9.6-27.6 months) and 9.6 months (6-18 months). The 2-year OS of those with indolent, aggressive, and lymphoblastic lymphoma was, respectively, 57%, 42%, and 41%. Patients with indolent lymphoma tended to have a better survival (P = .131, log rank test; P = .064, G. Wilcoxon test) (Figure 3). Kaplan-Meier estimates of OS of patients with 4 histologic subtypes of aggressive lymphoma, including diffuse large B-cell lymphoma (n = 44), PTCL (n = 22), extranodal NK/T-cell lymphoma, nasal type (n = 19), and others (n = 26), are shown in Figure 4.

The 14 clinical factors shown in Table 4 were assessed with regard to their relation to OS. A univariate analysis revealed that 5 factors, including chemoresistant disease, prior autograft, prior radiotherapy, aggressive lymphoma other than PTCL, and clinical subtype (aggressive versus indolent), were associated with a significantly worse OS. In a multivariate analysis using Cox proportional hazard models, chemoresistant disease, prior autograft, and prior radiotherapy were associated with a worse OS (Table 4). Acute GVHD, which was treated as a time-dependent variable, was not a significant factor for OS in both univariate and multivariate models. The relation between OS and response to chemotherapy is shown in Figure 5.

Discussion

This report describes the general outcome of patients with NHL who underwent modern allo-HSCT with a myeloablative regimen

Table 2. Causes of treatment-related mortality

Causes of TRM	Patients, no. (%)	No. of patients with GVHD	No. of patients without GVHD	Early death, no.*
GVHD	11 (11)			
Infection	29 (30)	15	8	6
Interstitial pneumonitis	16 (17)	15	0	1
Venocclusive disease	11 (11)	5	4	2
Thrombotic microangiopathy	8 (8)	7	1	0
Heart failure	7 (7)	3	1	3
Hemorrhage	4 (4)	3	1	0
Renal failure	3 (3)	2	1	0
Others†	9 (9)	6	1	2
Total	98 (100)	56	17	14

GVHD indicates graft-versus-host disease.

*Early death was defined as treatment-related death before engraftment.

†Others (n = 9) were acute respiratory distress syndrome (n = 2), hepatic failure (n = 2), leukoencephalopathy (n = 1), secondary solid cancer (n = 1), suicide (n = 1), and unknown cause (n = 2).

Table 3. Univariate and multivariate analyses of treatment-related mortality

Variable	No.	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Age at transplantation			.035		—
Younger than 40 y	158	1.00		—	
40 y or older	75	1.82 (1.04-3.17)		—	
Clinical subtype			.349		—
Indolent	38	1.00		—	
Lymphoblastic	84	1.47 (0.66-3.32)		—	
Clinical subtype			.103		—
Indolent	38	1.00		—	
Aggressive	111	1.91 (0.88-4.16)		—	
Aggressive lymphoma			.045		—
PTCL	22	1.00		—	
Non-PTCL	89	2.85 (1.02-7.94)		—	
Response to chemotherapy			< .001		< .001
Sensitive	128	1.00		1.00	
Resistant	105	3.41 (1.97-5.88)		2.95 (1.66-5.25)	
Prior autograft			< .001		< .001
No	193	1.00		1.00	
Yes	40	4.74 (2.23-10.07)		4.09 (1.85-9.04)	
Prior radiotherapy			.010		—
No	152	1.00		—	
Yes	81	2.05 (1.18-3.55)		—	
Years of transplantation			.225		—
1996-2001	187	1.00		—	
1990-1995	46	1.49 (0.78-2.86)		—	
Donor			.295		—
HLA-matched	197	1.00		—	
HLA-mismatched	36	1.46 (0.72-2.98)		—	
HLA-matched donor			.437		—
Related	154	1.00		—	
Unrelated	43	1.24 (0.72-2.15)		—	
Source of stem cells*			.544		—
BM	159	1.00		—	
PBSCs	70	1.09 (0.82-1.46)		—	
Conditioning regimen			.144		—
TBI-containing	193	1.00		—	
Others	40	1.67 (0.84-3.30)		—	
GVHD prophylaxis†			.169		—
Cyclosporin + methotrexate	204	1.00		—	
Tacrolimus + methotrexate	22	1.86 (0.77-4.51)		—	
Acute GVHD			.537		—
No	78	1.00		—	
Yes	155	1.19 (0.69-2.06)		—	
Chronic GVHD			< .001		.029
No	79	1.00		1.00	
Yes	154	2.76 (1.53-4.98)		2.02 (1.07-3.77)	

CI indicates confidence interval; PTCL, peripheral T-cell lymphoma; HLA, human leukocyte antigen; BM, bone marrow; GVHD, graft-versus-host disease; and —, not applicable.

*Those who received cord blood (n = 2) or BM + PBSC (n = 2) were excluded because of the small number of patients.

†Seven patients using other GVHD prophylaxis were excluded.

in Japan, focusing on the background problems of myeloablative therapy and the identification of risk factors for TRM and OS. We showed that long-term, lymphoma-free survival could be achieved in approximately 40% of patients. Patients with FL had a better prognosis, consistent with previous reports.^{8,10} Even in patients with aggressive lymphoma or LBL, long-term survival of 35% was identified, consistent with previous reports.^{8,9} However, there were no significant differences between clinical subtypes (eg, aggressive versus indolent or PTCL versus non-PTCL) in a multivariate analysis. Because rituximab became commercially available after 2001 in Japan, patients with B-cell NHL who received anti-CD20

antibody therapy were not included in this study. The clinical effect of the introduction of rituximab on outcome after allogeneic transplantation should be carefully evaluated in a future study.

Our study confirmed a high TRM rate (42%) after conventional allo-HSCT with a myeloablative regimen, consistent with previous reports.^{4-8,25} One of the major causes of death was severe regimen-related toxicities, which included interstitial pneumonitis, venoocclusive disease, cardiac and renal toxicity, and organ hemorrhage. Although TBI-based regimens are frequently chosen because lymphoma cells are considered to be sensitive to irradiation, they have also been associated with long-term complications, including

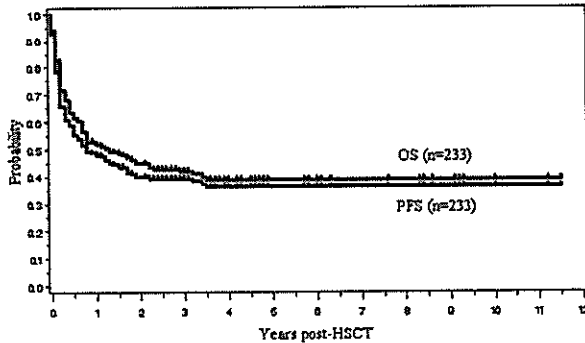


Figure 2. Overall survival (OS) and progression-free survival (PFS) for all 233 patients.

interstitial pneumonitis.^{26,27} Because most patients received TBI-based regimens as reported,^{4,5,7} we failed to detect any significant differences in TRM between those who received or did not receive TBI.

Another major cause of death in our study was GVHD and/or infection. Of the 98 patients who died of treatment-related complications in our study, 29 (30%) died of infection. At least half of the patients (15 of 29) who died of infectious complications also had GVHD. In a prospective trial of allo-HSCT for patients with NHL, infection accounted for 63% of all TRM,²⁸ whereas other studies, including ours, have reported an incidence of 25% to 30%.^{4,6} In practical transplantation procedures, complications are usually multifactorial, and it is always very difficult to define the exact cause of death, which may account for the wide variations in the incidence of infections among those who died of TRM (18%-63%) in previous reports.^{4,5,28,29}

In this study, the incidence of chronic GVHD was high (48%), and chronic GVHD was a risk factor for TRM. The reason for the higher incidence of chronic GVHD in our study compared with the IBMTR report^{9,30} was that the IBMTR study included data of patients who died within 100 days after allo-HSCT, whereas we excluded these patients. Unexpectedly, the incidence of chronic GVHD was higher in patients who had GVHD prophylaxis with tacrolimus plus methotrexate than in those with cyclosporin plus methotrexate. In Japan, there is a clear tendency to select tacrolimus rather than cyclosporine for GVHD prophylaxis in unrelated or HLA-mismatched transplantation.^{31,32} In addition, PBSCT is not yet permitted for unrelated transplantation. Altogether, the higher

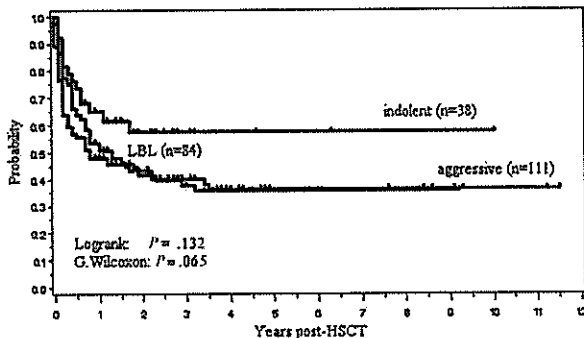


Figure 3. Overall survival stratified according to the clinical subtype. Indolent lymphoma included all grades of FL and extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue. Aggressive lymphoma included all lymphomas except for indolent and lymphoblastic lymphoma (LBL).

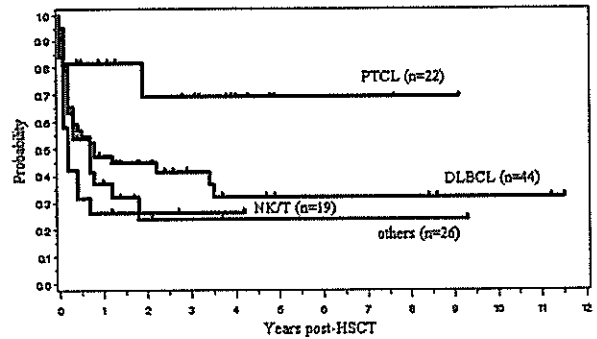


Figure 4. Overall survival for patients with 4 histologic subtypes of aggressive lymphoma. PTCL indicates peripheral T-cell lymphoma, unspecified; DLBCL, diffuse large B-cell lymphoma; NK/T, extranodal NK/T-cell lymphoma, nasal type.

incidence of GVHD observed in the tacrolimus group may simply reflect that patients with a higher risk of GVHD were selected to receive tacrolimus.

We found that the incidence of disease relapse/progression of NHL was low (21%). High TRM in the early phase of the transplantation course may mask later disease relapse/progression, and this made it difficult to estimate the relapse rate in this study. OS and PFS were not affected by the severity of acute GVHD. Our limited analysis failed to confirm a GVL effect after myeloablative allo-HSCT. Although the risk of relapse for patients with acute or chronic GVHD was not significantly different from that of patients without acute or chronic GVHD in previous studies with malignant lymphoma,^{8,10,30} a study from the Japan Marrow Donor Program showed that the development of grade II to IV acute GVHD was associated with a lower incidence of disease progression after unrelated HSCT.³¹ It has been reported that a low level of acute GVHD was associated with improved OS, and all levels of acute GVHD were associated with a decrease in the relapse rate for intermediate-grade NHL.⁸ High levels of acute GVHD had a deleterious effect on OS but were associated with an improved relapse rate for LBL.⁸ Thus, our study confirmed that greater effort is required to reduce GVHD-related complications after myeloablative allo-HSCT.

We confirmed that chemoresistance before allo-HSCT and prior autograft were significant risk factors for both OS and TRM. RIST or a less organ-toxic myeloablative allo-HSCT using a combination of fludarabine plus intravenous busulfan may be applied more safely in this population to reduce TRM.^{19-21,33,34} However, further studies are needed to determine whether reduced-intensity conditioning could control activity of chemoresistant disease. In contrast to previous studies, we showed that prior radiotherapy was associated with a significantly worse OS, which may be related to the fact that 44 (54%) of the 81 patients who had a history of local radiotherapy had refractory disease at transplantation. Hence, it might be that prior radiotherapy was a marker of survival for more advanced and refractory disease.

In conclusion, we confirmed that myeloablative allo-HSCT is a curative therapeutic option in a subset of patients with NHL, but it carries a high risk of toxicities and TRM. Chemoresistant disease and a history of previous autograft are risk factors for both OS and TRM. Whether the introduction of a reduced-intensity transplantation procedure results in reduction of TRM should be evaluated, and more effective GVHD prophylaxis while maintaining a GVL effect should be developed.

Table 4. Univariate and multivariate analyses of overall survival

Variable	No.	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Age at transplant			.134	—	—
Younger than 40 y	158	1.00		—	—
40 y or older	75	1.32 (0.92-1.90)		—	—
Clinical subtype			.126	—	—
Indolent	38	1.00		—	—
Lymphoblastic	84	1.57 (0.88-2.80)		—	—
Clinical subtype			.045	—	—
Indolent	38	1.00		—	—
Aggressive	111	1.77 (1.01-3.11)		—	—
Aggressive lymphoma			.004	—	—
PTCL	22	1.00		—	—
Non-PTCL	89	3.45 (1.47-7.69)		—	—
Response to chemotherapy			< .001	—	—
Sensitive	128	1.00		—	—
Resistant	105	3.31 (2.30-4.76)		3.12 (2.16-4.51)	< .001
Prior autograft			< .001	—	—
No	193	1.00		—	—
Yes	40	2.59 (1.73-3.87)		2.18 (1.43-3.30)	< .001
Prior radiotherapy			< .001	—	—
No	152	1.00		—	—
Yes	81	1.99 (1.41-2.83)		1.47 (1.02-2.11)	.037
Years of transplantation			.932	—	—
1996-2001	187	1.00		—	—
1990-1995	46	1.02 (0.67-1.54)		—	—
Donor			.076	—	—
HLA-matched	197	1.00		—	—
HLA-mismatched	36	1.50 (0.96-2.33)		—	—
HLA-matched donor			.769	—	—
Related	154	1.00		—	—
Unrelated	43	0.93 (0.58-1.50)		—	—
Source of stem cells*			.095	—	—
BM	159	1.00		—	—
PBSCs	70	1.37 (0.95-2.00)		—	—
Conditioning regimen			.107	—	—
TBI-containing	193	1.00		—	—
Others	40	1.42 (0.93-2.17)		—	—
GVHD prophylaxis†			.227	—	—
Cyclosporin + methotrexate	204	1.00		—	—
Tacrolimus + methotrexate	22	1.40 (0.81-2.40)		—	—
Acute GVHD-time‡			.264	1.28 (0.87-1.90)	.213

CI indicates confidence interval; PTCL, peripheral T-cell lymphoma; HLA, human leukocyte antigen; BM, bone marrow; GVHD, graft-versus-host disease; and —, not applicable.

*Those who received cord blood (n = 2) or BM + PBSCs (n = 2) were excluded because of the small number of patients.

†Seven patients using other GVHD prophylaxis were excluded.

‡Acute GVHD was treated as time-dependent variable.

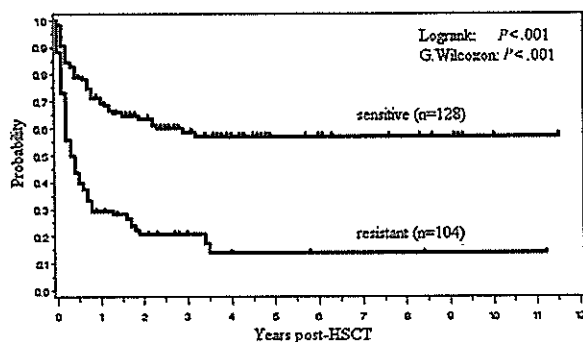


Figure 5. The relation between overall survival and response to chemotherapy.

Acknowledgments

We thank the many physicians in the participating teams who contributed to this survey.

Appendix

The following institutions contributed data to this study: Asahikawa Medical College Hospital, Hokkaido University Hospital, Sapporo Hokuyu Hospital, Akita University Hospital, Gunma Saiseikai Maebashi Hospital, Jichi Medical School Hospital, Suifu Hospital, Saitama Cancer Center Hospital, Jikei University Kashiwa Hospital, Chiba Aoba Municipal

Hospital, Tokyo Metropolitan Komagome Hospital, National Cancer Center Hospital, Keio University Hospital, Toranomon Hospital, Yokohama City University Hospital, Kanagawa Cancer Center, Tokai University Hospital, Kurobe City Hospital, Kanazawa University Hospital, Ishikawa Prefectural Central Hospital, Nagoya City University Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, JA Aichi Showa Hospital, Kyoto University Hospital, Kyoto Prefectural University of Medicine Hospital, Osaka University Hospital, Osaka City University Hospital, Kansai Medical University Hospital, Kinki University Hospital, Osaka Medical Center for Cancer and

Cardiovascular Diseases, Osaka City General Hospital, Rinku General Medical Center Izumisano Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Okayama University Hospital, Okayama Medical Center, Shimane Prefectural Central Hospital, Takamatsu Red Cross Hospital, Ehime Prefectural Central Hospital, University of Occupational and Environmental Health Hospital, Kitakyushu Municipal Medical Center, Kyushu Cancer Center, Kokura Memorial Hospital, Fukuoka University Hospital, Hamanomachi Hospital, Harasanshin Hospital, Saga Prefectural Hospital Koseikan, Sasebo Municipal General Hospital, Miyazaki Prefectural Miyazaki Hospital, Imamura Bun-in Hospital, and Ryukyuu University Hospital.

References

- Phillip T, Guglielmi C, Hagenbeek A, et al. Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. *N Engl J Med*. 1995;333:1540-1545.
- Milpied N, Deconinck E, Gaillard F, et al. Initial treatment of aggressive lymphoma with high-dose chemotherapy and autologous stem-cell support. *N Engl J Med*. 2004;350:1287-1295.
- Horning SJ, Negrin RS, Hoppe RT, et al. High-dose therapy and autologous bone marrow transplantation for follicular lymphoma in first complete or partial remission: results of a phase II clinical trial. *Blood*. 2001;97:404-409.
- Dhedini N, Giraudier S, Gaulard P, et al. Allogeneic bone marrow transplantation in aggressive non-Hodgkin's lymphoma (excluding Burkitt and lymphoblastic lymphoma): a series of 73 patients from the SFGM database. *Br J Haematol*. 1999;107:154-161.
- van Besien KW, Mehra RC, Giralt SA, et al. Allogeneic bone marrow transplantation for poor-prognosis lymphoma: response, toxicity and survival depend on disease histology. *Am J Med*. 1996;100:299-307.
- van Besien K, Thall P, Korbiling M, et al. Allogeneic transplantation for recurrent or refractory non-Hodgkin's lymphoma with poor prognostic features after conditioning with thiolepa, busulfan, and cyclophosphamide: experience in 44 consecutive patients. *Biol Blood Marrow Transplant*. 1997;3:150-156.
- van Besien K, Sobocinski KA, Rowings PA, et al. Allogeneic bone marrow transplantation for low-grade lymphoma. *Blood*. 1998;92:1832-1836.
- Peniket AJ, Ruiz de Elvira MC, Taghipour G, et al. An EBMT registry matched study of allogeneic stem cell transplants for lymphoma: allogeneic transplantation is associated with a lower relapse rate but a higher procedure-related mortality rate than autologous transplantation. *Bone Marrow Transplant*. 2003;31:667-678.
- Levine JE, Harris RE, Loberiza FR Jr, et al. A comparison of allogeneic and autologous bone marrow transplantation for lymphoblastic lymphoma. *Blood*. 2003;101:2476-2482.
- van Besien K, Loberiza FR Jr, Bajorunaitė R, et al. Comparison of autologous and allogeneic hematopoietic stem cell transplantation for follicular lymphoma. *Blood*. 2003;102:3521-3529.
- Forrest DL, Thompson K, Nevill TJ, Couban S, Fernandez LA. Allogeneic hematopoietic stem cell transplantation for progressive follicular lymphoma. *Bone Marrow Transplant*. 2002;29:973-978.
- Berdeja JG, Jones RJ, Zahurak ML, et al. Allogeneic bone marrow transplantation in patients with sensitive low-grade lymphoma or mantle cell lymphoma. *Biol Blood Marrow Transplant*. 2001;7:561-567.
- Stein RS, Greer JP, Goodman S, et al. Limited efficacy of intensified preparative regimens and autologous transplantation as salvage therapy in high-grade non-Hodgkin's lymphoma. *Leuk Lymphoma*. 2001;40:521-528.
- Khouri IF, Lee MS, Romaguera J, et al. Allogeneic hematopoietic transplantation for mantle-cell lymphoma: molecular remissions and evidence of graft-versus-malignancy. *Ann Oncol*. 1999;10:1293-1299.
- Zaja F, Russo D, Silvestri F, et al. Retrospective analysis of 23 cases with peripheral T-cell lymphoma, unspecified: clinical characteristics and outcome. *Haematologica*. 1997;82:171-177.
- Makita M, Maeda Y, Takenaka K, et al. Successful treatment of progressive NK cell lymphoma with allogeneic peripheral stem cell transplantation followed by early cyclosporine tapering and donor leukocyte infusions. *Int J Hematol*. 2002;76:94-97.
- Khouri IF, Lee MS, Saliba RM, et al. Nonablative allogeneic stem-cell transplantation for advanced/recurrent mantle-cell lymphoma. *J Clin Oncol*. 2003;21:4407-4412.
- Khouri IF, Saliba RM, Giralt SA, et al. Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. *Blood*. 2001;98:3595-3599.
- Tanimoto TE, Kusumi E, Hamaki T, et al. High complete response rate after allogeneic hematopoietic stem cell transplantation with reduced-intensity conditioning regimens in advanced malignant lymphoma. *Bone Marrow Transplant*. 2003;32:131-137.
- Bierman PJ. Allogeneic bone marrow transplantation for lymphoma. *Blood Rev*. 2000;14:1-13.
- Jaffe ES, Harris NL, Stein H, et al, eds. *World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2001.
- Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15:825-828.
- Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med*. 1980;69:204-217.
- Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *Am Stat Assoc J*. 1958;457-481.
- Akpek G, Ambinder RF, Piantadosi S, et al. Long-term results of blood and marrow transplantation for Hodgkin's lymphoma. *J Clin Oncol*. 2001;19:4314-4321.
- Borlin MM, Kay HE, Gale RP, Rimm AA. Factors associated with interstitial pneumonitis after bone-marrow transplantation for acute leukaemia. *Lancet*. 1982;1:437-439.
- Weiner RS, Borlin MM, Gale RP, et al. Interstitial pneumonitis after bone marrow transplantation. Assessment of risk factors. *Ann Intern Med*. 1986;104:168-175.
- Ratanatharathorn V, Uberti J, Karanes C, et al. Prospective comparative trial of autologous versus allogeneic bone marrow transplantation in patients with non-Hodgkin's lymphoma. *Blood*. 1994;84:1050-1055.
- Chopra R, Goldstone AH, Pearce R, et al. Autologous versus allogeneic bone marrow transplantation for non-Hodgkin's lymphoma: a case-controlled analysis of the European Bone Marrow Transplant Group Registry data. *J Clin Oncol*. 1992;10:1690-1695.
- Freytes CO, Loberiza FR, Rizzo JD, et al. Myeloablative allogeneic hematopoietic stem cell transplantation in patients who experience relapse after autologous stem cell transplantation for lymphoma: a report of the International Bone Marrow Transplant Registry. *Blood*. 2004;104:3797-3803.
- Izutsu K, Kanda Y, Ohno H, et al. Unrelated bone marrow transplantation for non-Hodgkin lymphoma: a study from the Japan Marrow Donor Program. *Blood*. 2004;103:1955-1960.
- Yanada M, Emi N, Naoe T, et al. Tacrolimus instead of cyclosporine used for prophylaxis against graft-versus-host disease improves outcome after hematopoietic stem cell transplantation from unrelated donors, but not from HLA-identical sibling donors: a nationwide survey conducted in Japan. *Bone Marrow Transplant*. 2004;34:331-337.
- de Lima M, Couriel D, Thall PF, et al. Once-daily intravenous busulfan and fludarabine: clinical and pharmacokinetic results of a myeloablative, reduced-toxicity conditioning regimen for allogeneic stem cell transplantation in AML and MDS. *Blood*. 2004;104:857-864.
- Russell JA, Tran HT, Quinlan D, et al. Once-daily intravenous busulfan given with fludarabine as conditioning for allogeneic stem cell transplantation: study of pharmacokinetics and early clinical outcomes. *Biol Blood Marrow Transplant*. 2002;8:468-476.

Monitoring of WT1-specific cytotoxic T lymphocytes after allogeneic hematopoietic stem cell transplantation

Yuriko Morita^{1,2}, Yuji Heike¹, Mami Kawakami¹, Osamu Miura², Shin-ichi Nakatsuka³, Michiko Ebisawa⁴, Shin-ichiro Mori¹, Ryuji Tanosaki¹, Takahiro Fukuda¹, Sung-Won Kim¹, Kensei Tobinai¹ and Yoichi Takaue^{1*}

¹Division of Hematology and Stem Cell Transplantation, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan

²Department of Hematology and Oncology, Graduate School of Medicine, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

³Department of Clinical Laboratory, Osaka Minami Medical Center, National Hospital Organization, Kawachi-nagano, Osaka, Japan

⁴SRL, Inc., Tokyo, Japan

Donor-derived cytotoxic T lymphocytes (CTL) that respond to tumor antigens emerge after hematopoietic stem cell transplantation (HSCT), particularly in association with the status of immune recovery. To analyze the frequency of CTL against PR1, PRAME and WT1 after HSCT, a tetramer-based analysis was performed in 97 samples taken from 35 patients (9 AML, 11 MDS, 2 CML, 4 ALL, 7 lymphoma and 2 renal cell carcinoma (RCC)) with the HLA-A02 phenotype. Regarding PR1, only 1 sample showed the presence of tetramer-positive cells (0.04%/lymphocyte). Similarly, in PRAME, only 10 of 97 samples were sporadically positive with low titers. For WT1, positive results were detected in 39 of 97 samples and 7 (2 CML, 1 ALL, 2 lymphoma and 2 RCC) patients clearly showed positive results more than once. On the basis of these results, we performed serial analyses of WT1-specific CTL during the clinical course in 2 patients with RCC, who underwent HSCT with a reduced-intensity regimen, to examine the precise correlation between the kinetics of CTL, the occurrence of GVHD and the observed clinical response. A higher positive rate for WT1-specific CTL and a correlation with the clinical response suggest that WT1 may be a useful antigen for a wider monitoring application.

© 2006 Wiley-Liss, Inc.

Key words: WT1; cytotoxic T lymphocytes; allogeneic hematopoietic stem cell transplantation; renal cell carcinoma

Allogeneic hematopoietic stem cell transplantation (HSCT) is considered to be the most powerful and distinguished immunotherapy since the concept of graft-versus-leukemia/tumor (GVL/GVT) effect has been established.¹ However, the underlying mechanism of the anticancer effect is not yet clear. Although the generation of donor-derived cytotoxic T lymphocytes (CTLs) in response to tumor antigens is considered to be the primary reason,^{2,3} the potential of any tumor-specific antigens to induce CTL should be critically evaluated in correlation with the clinical response. It is very likely that the immunogenic antigen, which plays the predominant role in the GVT effect, will be a potent candidate antigen for clinically realistic immunotherapy, including tumor vaccine, dendritic cell therapy and adoptive CTL infusion, to treat malignant disorders. WT1, PR1 and PRAME have been attractive targets for immunotherapy because of their expression in a wide variety of tumors, with a relative lack of expression in most normal tissues.^{4–6}

One of the Wilms' tumor genes, WT1, encodes a zinc finger transcription factor,⁷ and binds to the early growth response-1 DNA consensus sequence in growth factor gene promoters.⁸ WT1 is expressed at a high level in most types of leukemia⁹ and various types of solid tumors, including melanoma, renal cell carcinoma (RCC), and lung, breast, testicular and ovarian cancer.^{10,11} Although WT1 is expressed at low amounts in the nuclei of some normal cells, it is limited to very few tissues, including splenic capsule and stroma, some gonadal cells and hematopoietic precursor cells.¹² It has been reported that MHC class I-restricted, WT1-specific CTLs were generated from human peripheral blood mononuclear cells (PBMC) by *in vitro* stimulation with WT1 peptide.^{13–15} Additionally, an anticancer effect of WT1-specific CTL and the rejection of WT1-expressing tumor cells have been reported.^{16–18}

The other candidates include PR1, which is derived from proteinase 3, a myeloid tissue-restricted serine protease present in azurophil granules in myeloid cells.⁵ PR1 is overexpressed in some leukemia cells, and it has been reported that PR1-specific CTL selectively lyses chronic myelogenous leukemia (CML).¹⁹ PRAME (preferentially expressed antigen in melanoma) is encoded by genes that are overexpressed in a wide variety of tumors, including melanoma (95% of patients), RCC (41%), lung cancer (50%), acute leukemia (30%) and multiple myeloma (52%).²⁰ PRAME is not expressed in normal tissue, except for testis, and very low levels are found in endometrium, ovaries and adrenals.^{6,20–23}

In this study, we assessed the frequency of CTL against WT1, PR1 and PRAME by tetramer assay in peripheral blood taken from patients who underwent HSCT, to identify the best candidate antigen for clinically applicable immunotherapy.

Material and methods

Subjects

After we obtained written informed consent, peripheral blood samples were obtained from patients with an HLA-A02 serotype who had undergone allogeneic HSCT, with a conventional (CST) or reduced-intensity regimen (RIST), between August 2000 and May 2004 at National Cancer Center Hospital (Tokyo, Japan). Patients who received non-T-cell depleted-HSCT from a serologically full HLA-matched donor for hematological malignancy or RCC were eligible for this study.

Samples were taken at least 3 weeks after transplantation with confirmed hematological engraftment and more than 90% donor chimerism. Additional requirements for sampling included complete remission in leukemia patients and disease without bone marrow involvement in those with lymphoma or RCC.

Tumor antigen epitope peptides and cytomegalovirus peptide

The following >80% pure HLA-A*0201 binding peptides were obtained for the experiments, using high-performance liquid chromatography (Qiagen, Tokyo): WT1 peptide RMFPNAPYL (amino acids [AA] 126–134), PR1 peptide VLQELNVTV (AA 169–177) and PRAME peptide ALYVDSLFFL (AA 300–309); cytomegalovirus (CMV) pp65 peptide NLVPMVATV (AA 495–503) was used as a positive control.

Tetramer staining

Antibodies to CD4, CD8, CD19, CD13, CD45RA, CD45RO, CD27, CD57, CCR7, PE-conjugated CMV-tetramer and PR1-tetramer were purchased from Beckman Coulter (Fullerton, CA), and

*Correspondence to: Department of Medical Oncology, National Cancer Center Hospital, 1-1 Tsukiji 5-Chome, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3/3248-1510. E-mail: ytakaue@ncc.go.jp
Received 30 October 2005; Accepted 1 February 2006
DOI 10.1002/ijc.21960
Published online 4 April 2006 in Wiley InterScience (www.interscience.wiley.com).

APC-conjugated CMV, WT1 and PRAME-tetramer were purchased from ProImmune (Oxford, UK). The antibodies and tetramer complexes were added to 200 μ L heparinized whole blood or cell suspension, and incubated for 15 min at room temperature in the dark. For the quantification of antigen-specific CTL, whole blood samples were used, and the red blood cells were lysed with ammonium chloride-based lysing solution after antibody staining. After being washed twice with BSA-containing PBS, the cells were fixed and analyzed on a flow cytometer (FACS Calibur, Becton Dickinson). Analysis was performed using Cellquest software. For the quantification of antigen-specific-CTL, whole blood samples were used, and CD4⁻, CD19⁻, CD13⁻, CD8⁺ and tetramer⁺ fraction of the lymphocyte gate were defined as antigen-specific CTL. Samples with more than 0.02% antigen-specific CTL per lymphocyte were defined as positive results. For immunophenotyping of the antigen-specific CTL, fresh PBMC, separated from heparinized blood by Ficoll-Hypaque (IBL, Japan) density gradient centrifugation, was used to acquire a higher number of lymphocytes (minimum of 10⁵) per analysis. The cells were gated on CD8⁺ and tetramer⁺ fraction of the lymphocyte, and then the positive ratios for CCR7, CD45RA, CD45RO, CD27 and CD57 were analyzed.

Expansion of antigen-specific CTL

PBMC was diluted at 1 \times 10⁶ cells/mL in RPMI 1640 (Sigma) supplemented with 10% FBS, gentamicin and streptomycin (hereafter referred to as culture medium). PBMC (2 \times 10⁶ cells) were seeded in a 24-well plate, and the peptide was added to a final concentration of 5 μ M on day 0. The peptide was diluted to 10 mg/mL in dimethyl sulfoxide (DMSO) prior to use, and the same amount of DMSO alone was used as a negative control. The culture was fed on days 4 and 7 by replacing half of the medium with a fresh culture medium containing 20 U/mL IL-2 and 10 μ M peptide. Cells were cultured for 14 days.

Intracellular cytokine staining

The cultured cells were washed with culture medium, and 5 \times 10⁴ cells per test were suspended in 200 μ L medium. Tetramer was added to the test samples, and incubated for 15 min at 37°C in the dark. For peptide-stimulated cells, specific peptides were added to a final concentration of 10 μ M and incubated for 6 hr at 37°C. Breferrdin A (Sigma; 10 μ g/mL) was added during the last 4 hr of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μ g/mL staphylococcal enterotoxin B or PBS. Samples were washed, permeabilized and stained with anti-IFN- γ and anti-CD8 antibodies, and analyzed using a FACS Calibur.

Genotyping of the HLA-A02 locus

The DNA genotype of the HLA-A02 locus was examined using a Micro SSP allele-specific HLA class I DNA typing tray (One Lambda, CA, USA), according to the manufacturer's protocol. In brief, DNA was extracted from the lymphocytes, and added to a PCR reaction buffer containing dNTP and Taq polymerase. The sample-reaction mixture was applied to a 96 PCR tray that had been preloaded with allele-specific primers for HLA-A02. After the PCR reaction, the samples were electrophoresed on a gel, and photographed by an UV transilluminator. HLA-genotype was determined by the patterns of the allele-specific PCR product.

Results

Tetramer assay

Samples were obtained from 35 patients (9 AML, 11 MDS, 2 CML, 4 ALL, 7 lymphoma and 2 RCC): 10 had received CST and 25 received RIST. The stem cell source was peripheral blood stem cells from a related donor in 24, related bone marrow in 2 and unrelated bone marrow in 9. The genotype for the HLA-A02 allele was analyzed in 27 patients: 13 had A*0201, 12 had

TABLE I - DETECTION OF ANTIGEN-SPECIFIC CTL BY TETRAMER ASSAY

Antigen	Disease	No. of patients	CTL-positive patients	No. of samples	Samples with 0.02% \leq CTL
WT1	AML	9	0	16	1
	MDS	11	0	17	2
	CML	2	2	8	4
	ALL	4	1	7	3
	NHL	7	2	14	7
	RCC	2	2	35	22
	Total	35	7 (20) ²	97	39 (40)
PRAME	AML	9	0	16	2
	MDS	11	0	17	2
	CML	2	0	8	0
	ALL	4	0	7	1
	NHL	7	0	14	0
	RCC	2	1	35	5
	Total	35	1 (2.8)	97	10 (10)
PR1	AML	9	0	16	1
	MDS	11	0	17	0
	CML	2	0	8	0
	ALL	4	0	7	0
	NHL	7	0	14	0
	RCC	2	0	8	0
	Total	35	0 (0)	70	1 (1.4)

¹When the patients showed 0.02% \leq CTL at least twice, they were considered to be positive. ²Values in parentheses indicate percentages.

A*0206, 1 had A*0207 and 1 had both A*0201 and A*0206 genotypes.

All samples from CMV-seropositive patients were positive for CMV-specific-CTL (CMV-CTL) with high titers (mean 2.3%), and the same result was obtained using CMV-tetramer purchased from Beckman Coulter or ProImmune. The frequency of CMV-CTL did not differ between the A*0201 and A*0206 genotypes, but the patient with the A*0207 genotype was negative for CMV-CTL, since he was seronegative for CMV.

Regarding WT1-specific CTL (WT1-CTL), 39 of 97 samples had more than 0.02% CTL per lymphocyte. Seven patients (2 CML, 1 ALL, 2 lymphoma and 2 RCC) showed positive results at least twice, and we defined them as WT1-CTL positive patients. Among those with positive WT1-CTL between days 40 and 520 postHSCT, 1 ALL patient and 1 CML patient received CST, while the other 5 patients received RIST. All of the WT1-CTL-positive patients had experienced skin involvement by graft-versus-host disease (GVHD) of grade 1-3 prior to the detection of WT1-CTL, except for 1 ALL patient. The other target organs of GVHD were the liver in 1 patient, and gut in 1 patient. The HLA-A*02 genotype in WT1-CTL-positive patients was A*0206 in 5, A*0201 in 1 and both the A*0201 and A*0206 genotypes in 1.

Regarding PR1, all 70 samples were 0%, except for 3 samples that showed 0.01 and 0.04%. The sample with 0.04% PR1-specific-CTL was taken from a patient with AML at day 925 postSCT. However, another sample taken at day 966 from the same patient was negative (0%), suggesting that the initial result was a false-positive one. Similarly, in PRAME, 87 of 97 samples were negative and 10 samples from 7 different leukemia, lymphoma or RCC patients were sporadically positive with low titers (<0.05%), but positive results were not found at different occasions. Only 1 RCC patient showed a positive result more than once. However, the staining of PRAME-tetramer was dull compared to the prominent positive staining of WT1-tetramer, which suggests that the result may have been false-positive (Table I).

Expansion of antigen-specific CTL

The samples taken from 7 patients (4 AML, 2 CML and 1 RCC) were cultured with WT1, PR1, PRAME, CMV peptide or DMSO. The frequency of antigen-specific-CTL was analyzed by tetramer assay before and 14 days after culture. The CMV-CTL expanded in all 7 samples by 2- to 50-fold, whereas none of the PR1 or PRAME

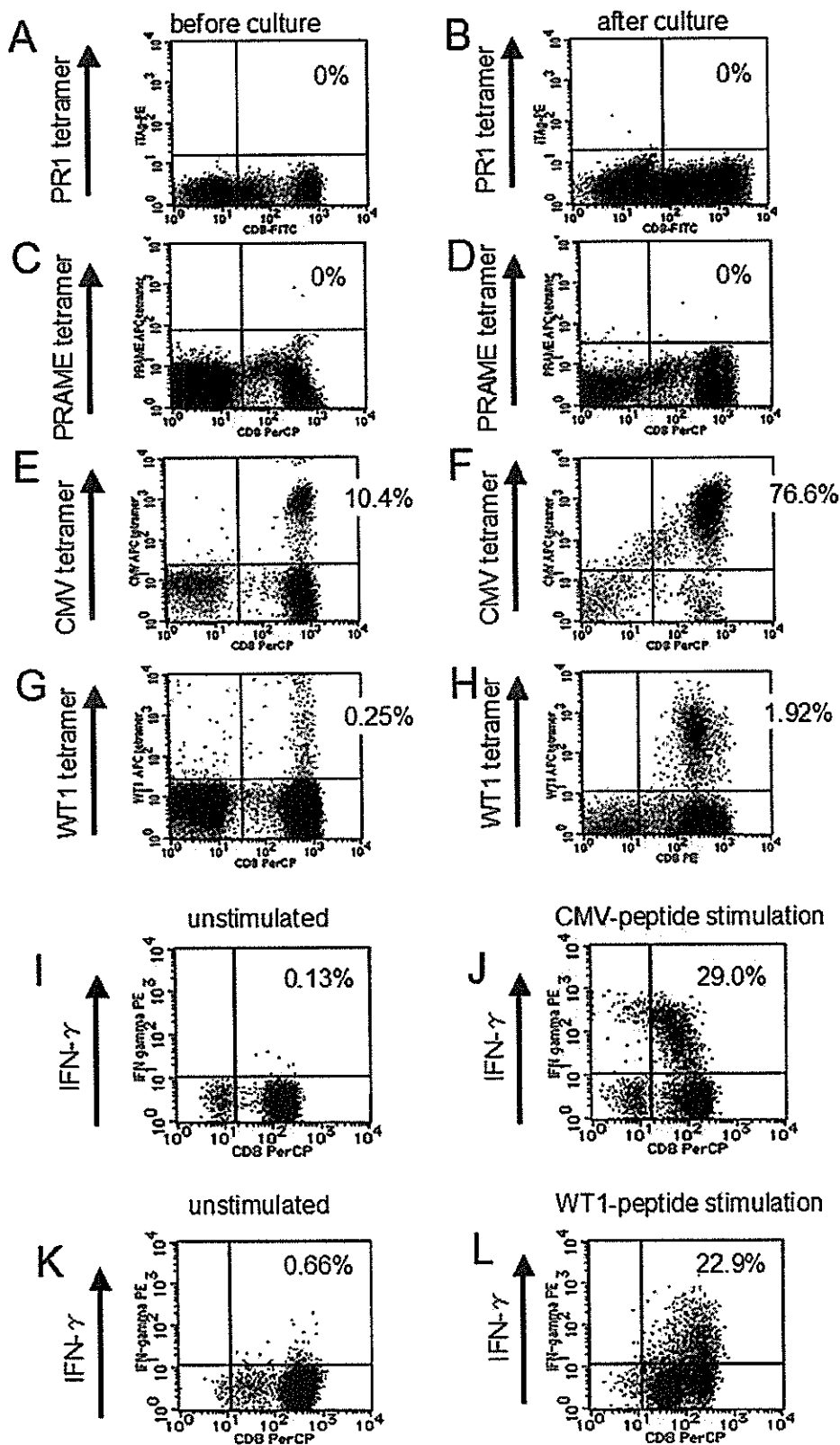


FIGURE 1 - Cell expansion of antigen-specific CTL and intracellular IFN- γ staining. PR1-CTL culture in CML patient (a, b) and PRAME-CTL culture in a RCC patient (c, d) remained in undetectable levels, even after expansion culture. CTL showed an expansion of CMV-CTL (e, f) and WT1-CTL (g, h). The intracellular IFN- γ staining showed that the CMV-CTL and WT1-CTL produced IFN- γ when stimulated with the peptide (j, l), while the unstimulated control did not (i, k). The cells are gated on a tetramer⁺ fraction.

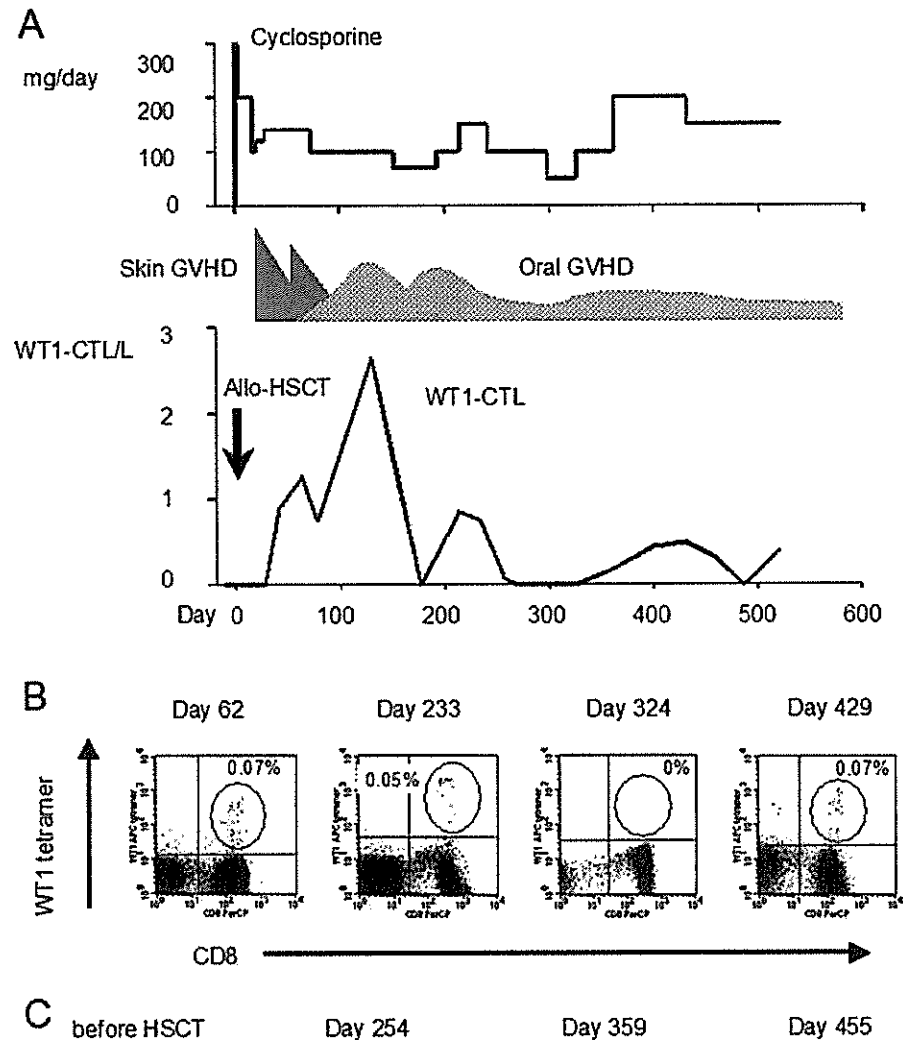


FIGURE 2 – Clinical course and tetramer analysis of Case 1 with RCC WT1-CTL was detected after the occurrence of skin GVHD, followed by a peak on day 128 when oral GVHD developed (*a, b*). Lung metastases slowly progressed while WT1-CTL disappeared (*c*).

peptide-containing cultures showed expansion of antigen-specific CTL, with all samples showing 0% for PR1-CTL and PRAME-CTL even after culture. The sample taken from a patient with RCC showed a meaningful expansion of WT1-CTL (Figs. 1*a–1h*).

Intracellular cytokine staining

The CMV-CTL and WT1-CTL, expanded by culture, were analyzed for intracellular IFN- γ . The cells were gated on tetramer-positive fraction of the lymphocyte gate, and the positive rate of CD8 and IFN- γ was analyzed. As for the CMV peptide cultured cells obtained from 7 patients, the mean rate of CD8⁺/IFN- γ ⁺ in the CMV-tetramer⁺ lymphocyte gate was 31.8% when stimulated with CMV peptide, whereas it was 1.72% when stimulated with PBS (negative control). A demonstrative result is shown in Figures 1*i* and 1*j*. For cells taken from a RCC patient and cultured with WT1 peptide, the rate of CD8⁺/IFN- γ ⁺ in the WT1-tetramer⁺ lympho-

cyte gate was 22.9% when stimulated with WT1 peptide, whereas it was 0.66% for negative control (Figs. 1*k* and 1*l*).

Serial analysis of WT1-CTL in patients with RCC

On the basis of Based on these results, we performed serial analyses of WT1-specific CTL during the clinical course of 2 patients with RCC who underwent RIST. The samples were obtained biweekly until day 200 and at longer intervals thereafter.

The first case is a 32-year-old female who had undergone resection of the primary disease, but had multiple lung metastases that were resistant to interferon therapy (Fig. 2). The histology of the primary disease was mixed RCC, which was positive for WT1 (Figs. 3*a* and 3*b*). She received RIST after conditioning with cladribine and busulfan, and cyclosporine (CSP) was administered as GVHD prophylaxis. Engraftment was achieved on day 12, which was followed by skin GVHD that extended to the whole

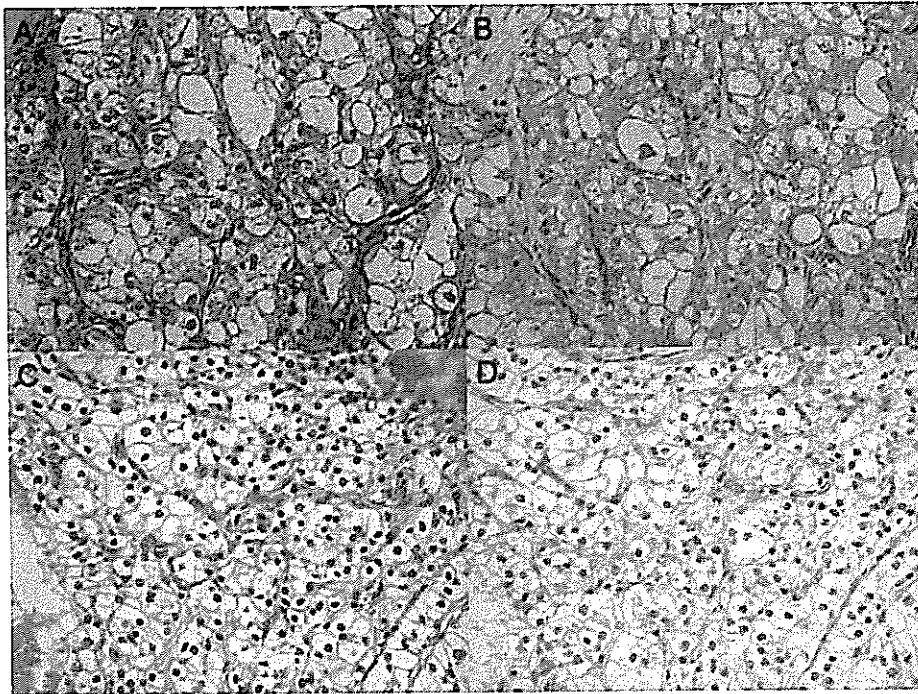


FIGURE 3 – Histology of resected RCC. The resected tumor sample in the first case with hematoxylin-eosin staining (a) confirmed a mixed cell carcinoma of the kidney. Immunostaining with WT1 (b) was positive for WT1. The second case with clear cell carcinoma (c) also showed positive staining for WT1 (d). Original magnification $\times 200$ for (a, b) and $\times 100$ for (c, d).

body. She was treated with topical corticosteroid after skin biopsy, which provided prompt resolution. WT1-CTL was detected at day 40 when the skin rash recurred, and the peak formation of WT1-CTL occurred on day 128 when oral chronic GVHD developed. The lung metastasis showed a stable disease until day 254, when the tumor started to grow with a slight improvement of oral GVHD, and WT1-CTL became undetectable on day 268. However, with a subsequent slight exaggeration of oral GVHD from day 359, a low titer of WT1-CTL once again became detectable from day 399. This patient is currently doing well at day 520 postHSCT, with a 24% increase in lung metastasis but with no new lesion.

The second case is a 43-year-old male patient who had the primary disease resected, but developed multiple lung metastases, which progressed despite interferon therapy. The histology of the primary disease was clear cell carcinoma that was positive for WT1 (Figs. 3c and 3d). The patient received RIST after conditioning with fludarabine busulfan, and anti-thymocyte globulin with CSP for GVHD prophylaxis (Fig. 4). He developed liver acute GVHD on day 83, after a rapid reduction in the dose of CSP. Liver GVHD was successfully treated by resuming CSP at a dose of 400 mg/body. He became positive for WT1-CTL on day 90; however, it disappeared along with the remission of liver GVHD. After CSP was tapered, skin GVHD occurred and WT1-CTL became detectable again. However, WT1-CTL disappeared from day 239 with the remission of skin GVHD, and the disease showed rapid progression. Donor lymphocyte infusion was performed on day 350 to induce a GVT effect, but WT1-CTL was not induced, and the patient died of respiratory failure because of disease progression on day 377 postHSCT.

Immunophenotype of WT1-CTL

The immunophenotype of the WT1-CTL in the RCC patients described earlier was analyzed (Fig. 5). The samples obtained at days 40, 77, 128, 149 and 233 posttransplantation from the first patient and at days 97, 146 and 196 in the second patient had adequate numbers of WT1-CTL for analysis. The phenotype did not differ significantly among samples taken from the same patient at different occasions. The WT1-CTL was effector phenotype in both patients, but different among the 2 patients as described later. In the

first patient, WT1-CTL was mainly effector memory phenotype. Seventy percent of the WT1-CTL expressed $CD45RA^+/CD45RO^-$ phenotype, 53% were of $CD57^+/CD45RO^-$ phenotype and 22% were of $CD57^-/CD45RO^-$ phenotype. In the other classification, 38% were $CD27^-/CD45RA^+$ and 34% were $CD27^+/CD45RA^+$. In the second patient, 80% of the WT1-CTL had the $CD45RA^-/CD45RO^+$ phenotype and 57% expressed $CD57^+/CD45RO^+$. In the other classification, 66% were $CD45RA^-/CD27^-$ and 21% were $CD45RA^-/CD27^+$. In both patients, over 95% of the WT1-CTL were negative for CCR7.

Discussion

Our study showed that CTL with avidity for the WT1 antigen are present in the peripheral blood of patients who underwent allogeneic HSCT for malignant disease. A GVT effect is thought to be mediated by expanding donor T cells, and a relationship has been reported between GVHD and disease control.²⁴ However, an optimal immune-monitoring system for tumor antigen-specific CTL, which is thought to be the effector cell for the GVT effect, has not been well established. Few studies on WT1-CTL have been reported, and most have focused on patients with leukemia²⁵ or those who received peptide vaccination.^{16,26} This is the first report of the kinetics of WT1-CTL in patients with RCC.

In the tetramer assay, we were able to reduce the background staining by sorting T cells with phenotypes, including CD4, CD13 and CD19, in addition to a lymphocyte gate using FSC and SSC. By this procedure, bright and distinct tetramer staining with fewer false-positive results was obtained, which made it possible to detect antigen-specific CTL present at very low levels. Tetramer assay using fresh peripheral blood is the best screening procedure, since it could be performed more easily and quickly than conventional procedures. Previous studies have used peptide stimulation and cytokine production analysis, such as ELISPOT assay or intracellular cytokine assay, to detect antigen-specific CTL.^{25,27} However, they are only semiquantitative, as it is impossible to exclude nonspecific cytokine production. We have overcome these problems by simultaneously staining the cells with tetramer and intracellular cytokine, which visualized the IFN- γ production pattern of antigen-specific CTL.

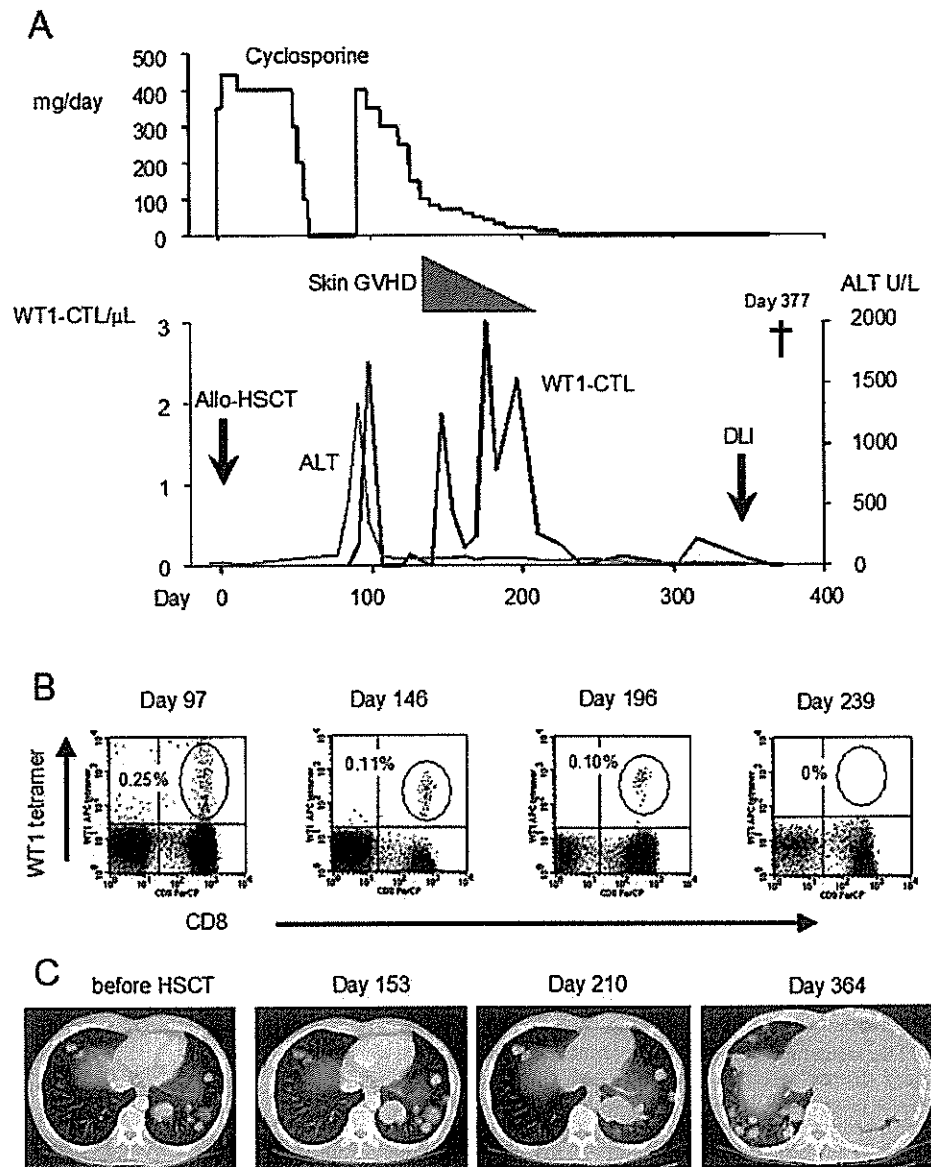


FIGURE 4 – Clinical course and tetramer kinetics in Case 2 with RCC. WT1-CTL emerged after the occurrence of liver and skin GVHD (*a, b*). The tumor rapidly grew after the disappearance of WT1-CTL (*c*).

The serial analysis of WT1 tetramer in 2 RCC patients clearly showed that WT1-CTL emerges after HSCT in relation with GVHD, and that they are associated with disease control/progression. It has been reported that the expansion of CD8⁺ IFN- γ -producing T cells and the incidence of GVHD are associated with the clinical response to nonmyeloablative allogeneic HSCT for RCC.² Our results suggest that WT1-CTL can be included among these CD8⁺ IFN- γ -producing T cells.

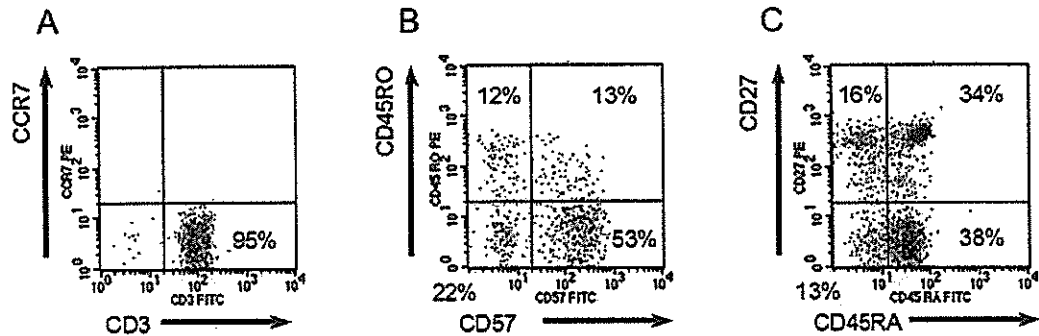
The immunostaining of WT1 showed a cytoplasmic pattern in both cases. Although WT1 is usually a nuclear protein, it is reported that some types of adenocarcinomas show cytoplasmic pattern.^{4,28} Also, a recent study showed that WT1 shuttles between the nucleus and cytoplasm, and thereby 10–50% of total cellular WT1 can be detected in the cytoplasm.²⁹ From these evidences, we conclude that the RCC cells in the 2 patients expressed WT1, which was present as a tumor antigen.

The WT1-CTL was detected in a relatively short period after HSCT, when the patient obtained full donor chimerism, which may suggest that the precursor of WT1-CTL was already present

in the donor graft. Since the WT1-CTL in the donor graft was under the level of detection of the tetramer assay and the WT1-CTL emerged soon after the occurrence of GVHD, it is quite likely that an immunological event associated with GVHD induced rapid expansion of the WT1-CTL. We can assume that GVHD drove the tumor-antigen to a peripheral circulation and stimulated WT1-CTL, together with a significant amount of cytokines, which were produced in the very early phase of HSCT and GVHD.

The immunophenotyping of antigen-specific CTL may be useful for predicting the function of CTL and disease prognosis.^{30–34} In RCC patients, most of the WT1-CTL detected was CCR7⁻/CD57⁺, consistent with an effector memory phenotype. The first case showed a relatively high frequency of CD45RA⁺/CD45RO⁻ phenotype with an equal ratio of CD27⁺ and CD27⁻, while most of the WT1-CTL in the second case had CD45RA⁻/CD45RO⁺ and CD27⁻ phenotypes. From a previous report, a cytokine-producing memory T cell subset capable of rapidly inducing IFN- γ and TNF- α synthesis shows the CD27⁻ phenotype, with varying degrees of CD45RA/CD45RO expression.³⁵ In another article, CTL with phe-

Case 1



Case 2

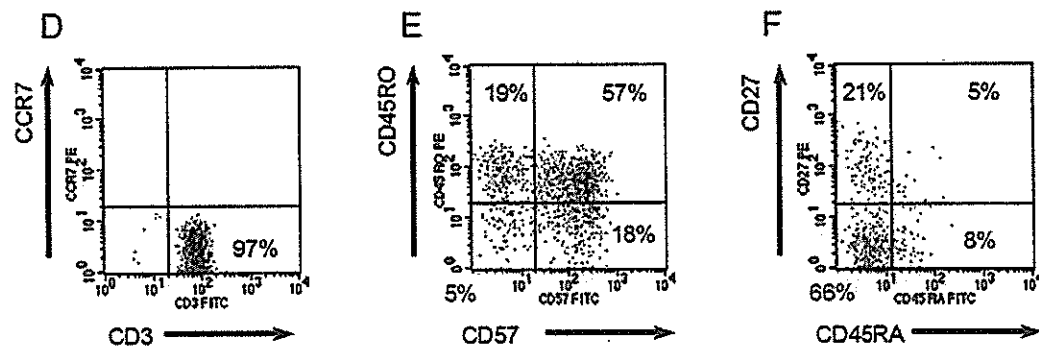


FIGURE 5 – Immunophenotype analysis of WT1-specific CTL. The immunophenotype of WT1-CTL in the first case of RCC was CCR7⁻ and mainly CD57⁺/CD45RO⁻/CD45RA⁺ (a-c). The immunophenotype of WT1-CTL in the second case was CCR7⁻ and CD57⁺/CD45RO⁺ and CD45RA⁻/CD27⁻ (d-f).

phenotypes, including CCR7⁻, CD45RA⁻ and CD45RO⁺, were shown to respond to antigen-specific peptide, while those with CCR7⁺, CD45RA⁺ and CD45RO⁻ phenotypes were associated with a lack of response to antigen-specific peptide.³⁶ Hence, based on the phenotype analysis in correlation with the clinical course, WT1-CTL seemed to have played a major role in disease control in the second patient, while an antitumor cell other than WT1-CTL may have had an effect in the first patient, since the disease progression in this patient was slow even when the WT1-CTL disappeared. NK cells may be the predominant antitumor cells, since this patient had a high proportion of NK cells in the peripheral blood at day 296 (7.0%/lymphocyte), which had been only 0.72% on day 149. Further analysis of antigen-specific CTL is critically required to elucidate the precise relationship between the phenotype and cell function.

We have previously demonstrated that CMV epitope NLVPMVATV is presented in both HLA-A*0201 and HLA-A*0206.³⁷ It is quite likely that WT1 epitope RMFPNAPYL is also commonly presented in HLA-A02 phenotypes, since WT1-CTL was detected not only in HLA-A*0201 patients but also in those with HLA-A*0206. Moni-

toring of WT1-CTL by tetramer assay can be widely applied to the HLA-A02 phenotype, since over 95% of HLA-A02 are either A*0201 or A*0206.^{38,39}

Although several studies on PR1-CTL detection in patients with leukemia have been reported,^{19,25,27} PR1- and PRAME-specific CTL were not detected in our study. We considered that one time positivity of the tetramer assay is not sufficient, since there may be an interassay variability. Since neither PR1-CTL nor PRAME-CTL was detected even after cell culture, in which the expansion of WT1-CTL and CMV-CTL was successful, we speculate that PR1-CTL and PRAME-CTL were not induced in most of the patients after HSCT.

In conclusion, our results suggest that WT1-CTL is involved in a GVT effect and WT1 is currently the best antigen for immunomonitoring after HSCT, while PR1 and PRAME are less potent antigens to be used for wider application. Although WT1-CTL may occur after HSCT *per se* without additional immunotherapy, it would be ideal to induce GVT effect with minimal GVHD. Further development of a WT1-based immunotherapy is desired to induce optimal antitumor immune response.

References

- Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. *Blood* 2004;103:767-76.
- Harlin H, Artz AS, Mahowald M, Rini BI, Zimmerman T, Vogelzang NJ, Gajewski TF. Clinical responses following nonmyeloablative allogeneic stem cell transplantation for renal cell carcinoma are associated with expansion of CD8⁺ IFN- γ -producing T cells. *Bone Marrow Transplant* 2004;33:491-7.
- Childs RW, Clave E, Tisdale J, Plante M, Hensel N, Barrett J. Successful treatment of metastatic renal cell carcinoma with a nonmyeloablative allogeneic peripheral-blood progenitor-cell transplant: evidence for a graft-versus-tumor effect. *J Clin Oncol* 1999;17:2044-9.
- Keilholz U, Menssen HD, Gaiger A, Menke A, Oji Y, Oka Y, Scheibenbogen C, Stauss H, Thiel E, Sugiyama H. Wilms' tumour gene 1 (WT1) in human neoplasia. *Leukemia* 2005;19:1318-23.
- Dengler R, Munstermann U, al-Batran S, Hausner I, Faderl S, Nerl C, Emmerich B. Immunocytochemical and flow cytometric detection of proteinase 3 (myeloblastin) in normal and leukaemic myeloid cells. *Br J Haematol* 1995;89:250-7.

6. Ikeda H, Lethe B, Lehmann F, van Baren N, Baurain JF, de Smet C, Chambost H, Vitale M, Moretta A, Boon T, Coulie PG. Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. *Immunity* 1997;6:199-208.
7. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeager H, Lewis WH, Jones C, Housman DE. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 1990;60:509-20.
8. Rauscher FJ, III. The WT1 Wilms tumor gene product: a developmentally regulated transcription factor in the kidney that functions as a tumor suppressor. *FASEB J* 1993;7:896-903.
9. Miwa H, Beran M, Saunders GF. Expression of the Wilms' tumor gene (WT1) in human leukemias. *Leukemia* 1992;6:405-9.
10. Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, Soma T, Tatekawa T, Kawakami M, Asada M, Kishimoto T, Sugiyama H. Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. *Jpn J Cancer Res* 1999;90:194-204.
11. Campbell CE, Kuriyan NP, Rackley RR, Caulfield MJ, Tubbs R, Finke J, Williams BR. Constitutive expression of the Wilms tumor suppressor gene (WT1) in renal cell carcinoma. *Int J Cancer* 1998;78:182-8.
12. Park S, Schalling M, Bernard A, Maheswaran S, Shipley GC, Roberts D, Fletcher J, Shipman R, Rheinwald J, Demetri G, Griffin J, Minden M, et al. The Wilms tumour gene WT1 is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. *Nat Genet* 1993;4:415-20.
13. Bellantuono I, Gao L, Parry S, Marley S, Dazzi F, Apperley J, Goldman JM, Stauss HJ. Two distinct HLA-A*0201-presented epitopes of the Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL. *Blood* 2002;100:3835-7.
14. Savage P, Gao L, Vento K, Cowburn P, Man S, Steven N, Ogg G, McMichael A, Epenetos A, Goulmy E, Stauss HJ. Use of B cell-bound HLA-A2 class I monomers to generate high-avidity, allo-restricted CTLs against the leukemia-associated protein Wilms tumor antigen. *Blood* 2004;103:4613-15.
15. Koesters R, Linnebacher M, Coy JF, Germann A, Schwitalle Y, Findeisen P, von Knebel Doeberitz M. WT1 is a tumor-associated antigen in colon cancer that can be recognized by in vitro stimulated cytotoxic T cells. *Int J Cancer* 2004;109:385-92.
16. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, Elisseeva OA, Oji Y, Kawakami M, Ikegame K, Hosen N, Yoshihara S, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci USA* 2004;101:13885-90.
17. Gaiger A, Reese V, Disis ML, Cheever MA. Immunity to WT1 in the animal model and in patients with acute myeloid leukemia. *Blood* 2000;96:1480-9.
18. Gao L, Bellantuono I, Elsasser A, Marley SB, Gordon MY, Goldman JM, Stauss HJ. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood* 2000;95:2198-203.
19. Mollndrem JJ, Lee PP, Wang C, Felio K, Kantarjian HM, Champlin RE, Davis MM. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat Med* 2000;6:1018-23.
20. Kessler JH, Beckman NJ, Bres-Vloemans SA, Verdijk P, van Veelen PA, Kloosterman-Joosten AM, Vissers DC, ten Bosch GJ, Kester MG, Sijts A, Wouter Drijfhout J, Ossendorp F, et al. Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. *J Exp Med* 2001;193:73-88.
21. van Baren N, Chambost H, Ferrant A, Michaux L, Ikeda H, Millard I, Olive D, Boon T, Coulie PG. PRAME, a gene encoding an antigen recognized on a human melanoma by cytolytic T cells, is expressed in acute leukaemia cells. *Br J Haematol* 1998;102:1376-9.
22. Greiner J, Ringhoffer M, Taniguchi M, Li L, Schmitt A, Shiku H, Dohner H, Schmitt M. mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. *Int J Cancer* 2004;108:704-11.
23. Steinbach D, Viehmann S, Zintl F, Gruhn B. PRAME gene expression in childhood acute lymphoblastic leukemia. *Cancer Genet Cytogenet* 2002;138:89-91.
24. Childs R, Chernoff A, Contentin N, Bahceci E, Schrupp D, Leitman S, Read EJ, Tisdale J, Dunbar C, Linehan WM, Young NS, Barrett AJ. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med* 2000;343:750-8.
25. Rezvani K, Grube M, Brenchley JM, Sconocchia G, Fujiwara H, Price DA, Gostick E, Yamada K, Melenhorst J, Childs R, Hensel N, Douek DC, et al. Functional leukemia-associated antigen-specific memory CD8+ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation. *Blood* 2003;102:2892-900.
26. Mailander V, Scheibenbogen C, Thiel E, Letsch A, Blau IW, Keilholz U. Complete remission in a patient with recurrent acute myeloid leukemia induced by vaccination with WT1 peptide in the absence of hematological or renal toxicity. *Leukemia* 2004;18:165,166.
27. Scheibenbogen C, Letsch A, Thiel E, Schmitt A, Mailander V, Baerwolf S, Nagorsen D, Keilholz U. CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. *Blood* 2002;100:2132-7.
28. Foster MR, Johnson JE, Olson SJ, Allred DC. Immunohistochemical analysis of nuclear versus cytoplasmic staining of WT1 in malignant mesotheliomas and primary pulmonary adenocarcinomas. *Arch Pathol Lab Med* 2001;125:1316-20.
29. Niksic M, Slight J, Sanford JR, Caceres JF, Hastie ND. The Wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes. *Hum Mol Genet* 2004;13:463-71.
30. Mollet L, Sadat-Sowti B, Duntze J, Leblond V, Bergeron F, Calvez V, Katlama C, Debre P, Autran B. CD8hi+CD57+ T lymphocytes are enriched in antigen-specific T cells capable of down-modulating cytotoxic activity. *Int Immunol* 1998;10:311-23.
31. Mortarini R, Piris A, Maurichi A, Molla A, Bersani I, Bono A, Bartoli C, Santinami M, Lombardo C, Ravagnani F, Cascinelli N, Parmiani G, et al. Lack of terminally differentiated tumor-specific CD8+ T cells at tumor site in spite of antitumor immunity to self-antigens in human metastatic melanoma. *Cancer Res* 2003;63:2535-45.
32. Tomiyama H, Takata H, Matsuda T, Takiguchi M. Phenotypic classification of human CD8+ T cells reflecting their function: inverse correlation between quantitative expression of CD27 and cytotoxic effector function. *Eur J Immunol* 2004;34:999-1010.
33. Powell DJ, Jr, Dudley ME, Robbins PF, Rosenberg SA. Transition of late-stage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy. *Blood* 2005;105:241-50.
34. Maczek C, Berger TG, Schuler-Thurner B, Schultz ES, Hamann A, Dunbar PR, Cerundolo V, Steinkasserer A, Schuler G. Differences in phenotype and function between spontaneously occurring melan-A-, tyrosinase- and influenza matrix peptide-specific CTL in HLA-A*0201 melanoma patients. *Int J Cancer* 2005;115:450-5.
35. Kern F, Khatamzas E, Surel I, Frommel C, Reinke P, Waldrop SL, Picker LJ, Volk HD. Distribution of human CMV-specific memory T cells among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms. *Eur J Immunol* 1999;29:2908-15.
36. Dunbar PR, Smith CL, Chao D, Salio M, Shepherd D, Mirza F, Lipp M, Lanzavecchia A, Sallusto F, Evans A, Russell-Jones R, Harris AL, et al. A shift in the phenotype of melan-A-specific CTL identifies melanoma patients with an active tumor-specific immune response. *J Immunol* 2000;165:6644-52.
37. Morita Y, Hosokawa M, Ebisawa M, Sugita T, Miura O, Takaue Y, Heike Y. Evaluation of cytomegalovirus-specific cytotoxic T-lymphocytes in patients with the HLA-A(*)02 or HLA-A(*)24 phenotype undergoing hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2005;36:303-11.
38. Sette A, Sidney J. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* 1999;50:201-12.
39. Tokunaga K, Ishikawa Y, Ogawa A, Wang H, Mitsunaga S, Moriyama S, Lin L, Bannai M, Watanabe Y, Kashiwase K, Tanaka H, Akaza T, et al. Sequence-based association analysis of HLA class I and II alleles in Japanese supports conservation of common haplotypes. *Immunogenetics* 1997;46:199-205.



Endoscopic evaluation for cytomegalovirus enterocolitis after allogeneic haematopoietic stem cell transplantation

Y Kakugawa, M Kami, T Kozu, N Kobayashi, H Shoda, T Matsuda, Y Saito, I Oda, T Gotoda, S Mori, R Tanosaki, N Murashige, T Hamaki, S Mineishi, Y Takae, T Shimoda and D Saito

Gut 2006;55:895-896
doi:10.1136/gut.2005.087031

Updated information and services can be found at:
<http://gut.bmj.com/cgi/content/full/55/6/895>

These include:

Email alerting service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article

Topic collections

Articles on similar topics can be found in the following collections

Transplantation (287 articles)
Other imaging techniques (1504 articles)
Infection (393 articles)

Notes

To order reprints of this article go to:
<http://www.bmjournals.com/cgi/reprintform>

To subscribe to *Gut* go to:
<http://www.bmjournals.com/subscriptions/>

PostScript

LETTERS

Endoscopic evaluation for cytomegalovirus enterocolitis after allogeneic haematopoietic stem cell transplantation

Cytomegalovirus (CMV) disease and graft versus host disease (GVHD) are serious complications after allogeneic haematopoietic stem cell transplantation (allo-SCT).¹ As clinical manifestations overlap,² it is difficult to make an early and accurate diagnosis of CMV enterocolitis in patients with gastrointestinal GVHD. This study aimed to estimate the usefulness of endoscopic examination for CMV enterocolitis after allo-SCT.

Between 1999 and 2003, 425 patients received allo-SCT at the National Cancer Centre Hospital. Eighty seven patients with gastrointestinal symptoms underwent colonoscopy, and gastrointestinal GVHD was diagnosed in 75 patients. Thirty three of these patients with a median age of 46 years (range 4-67) who had persistent diarrhoea and/or abdominal pain despite optimal treatment underwent repeat colonoscopy. After informed consent was obtained, we examined from the terminal ileum to the rectum by colonoscopy. Biopsy specimens were obtained from severely involved areas. If we

could not detect abnormal findings, biopsy specimens were taken from normal appearing areas. Diagnosis of CMV enterocolitis and management of CMV reactivation have been reported previously.³ A univariate analysis using Fisher's exact test was performed to compare differences in endoscopic findings between patients with and without CMV enterocolitis. A *p* value of <0.05 was considered significant.

CMV enterocolitis was diagnosed in eight patients; the remaining 25 patients served as controls. Symptoms in patients with and without CMV enterocolitis, respectively, were abdominal pain (*n*=4 and *n*=12), bloody stool (*n*=1 and *n*=6), and watery diarrhoea (*n*=7 and *n*=25). All of the patients with CMV enterocolitis tested positive for CMV antigenaemia with median levels of 84.5 per 50 000 cells (range 8-932) when they underwent colonoscopy. Eight controls tested positive for CMV antigenaemia. Median levels were 0 per 50 000 cells (range 0-17).

For endoscopic findings, patients with and without CMV enterocolitis, respectively, showed erosion (*n*=5 and *n*=5), ulceration (*n*=1 and *n*=1), oozing (*n*=2 and *n*=5), redness (*n*=7 and *n*=15), oedema (*n*=1 and *n*=6), disappearance of vascular network (*n*=8 and *n*=21), atrophic villi (*n*=2 and *n*=4), rough mucosa (*n*=6 and *n*=18), and exfoliation of mucosa (*n*=3 and *n*=10) (fig 1). Erosion was more frequent in patients with CMV enterocolitis than in controls (*p*=0.036). Gastrointestinal GVHD was

diagnosed in seven patients with CMV enterocolitis and in all of the controls. A total of 46 biopsy specimens were obtained from the eight patients with CMV enterocolitis (table 1). CMV inclusion bodies were found in 18 specimens, most of which were obtained from erosions (*n*=13). CMV inclusion bodies were distributed widely from the terminal ileum to the rectum.

The present study suggested endoscopic findings such as erosions were a useful marker for early diagnosis of CMV enterocolitis. Most of the CMV inclusion bodies were obtained from erosions. In contrast, only one had punched out ulcerations which had been considered characteristic of CMV enterocolitis.⁴⁻⁶ In case No 7, the only patient with punched out ulcerations, three specimens obtained from ulcerations were negative for CMV inclusion bodies while one specimen obtained from erosions showed inclusion bodies. CMV infected vascular endothelium is considered to narrow the vessels and to induce local ischaemia and eventually erosions and ulcerations.⁷ The small size of the erosions compared with that of ulcerations may enable biopsy of the whole lesion and the high yield of CMV infected cells. Biopsy of erosions can be reasonably effective for early diagnosis of CMV enterocolitis.

Colonoscopies in transplant recipients are often restricted to the rectum and sigmoid colon to minimise colonoscopic invasion. If we had examined only the rectum and sigmoid colon, CMV enterocolitis in six of the eight patients would have been missed. Total colonoscopy is necessary to make an early diagnosis of CMV enterocolitis. The present study suggests the usefulness and necessity of colonoscopy for an early diagnosis of CMV enterocolitis after allo-SCT.

Y Kakugawa

Endoscopy Division, the National Cancer Centre Hospital, Tokyo, Japan

M Kami

Haematopoietic Stem Cell Transplantation Unit, the National Cancer Centre Hospital, Tokyo, Japan

T Kozu, N Kobayashi, H Shoda, T Matsuda, Y Saito, I Oda, T Gotoda

Endoscopy Division, the National Cancer Centre Hospital, Tokyo, Japan

S Mori, R Tanosaki, N Murashige, T Hamaki, S Mineishi, Y Takave

Haematopoietic Stem Cell Transplantation Unit, the National Cancer Centre Hospital, Tokyo, Japan

T Shimoda

Pathological Division, the National Cancer Centre Hospital, Tokyo, Japan

D Saito

Endoscopy Division, the National Cancer Centre Hospital, Tokyo, Japan

Correspondence to: Dr Y Kakugawa, Endoscopy Division, the National Cancer Centre Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; yakakuga@ncc.go.jp

doi: 10.1136/gut.2005.087031

Conflict of interest: None declared.

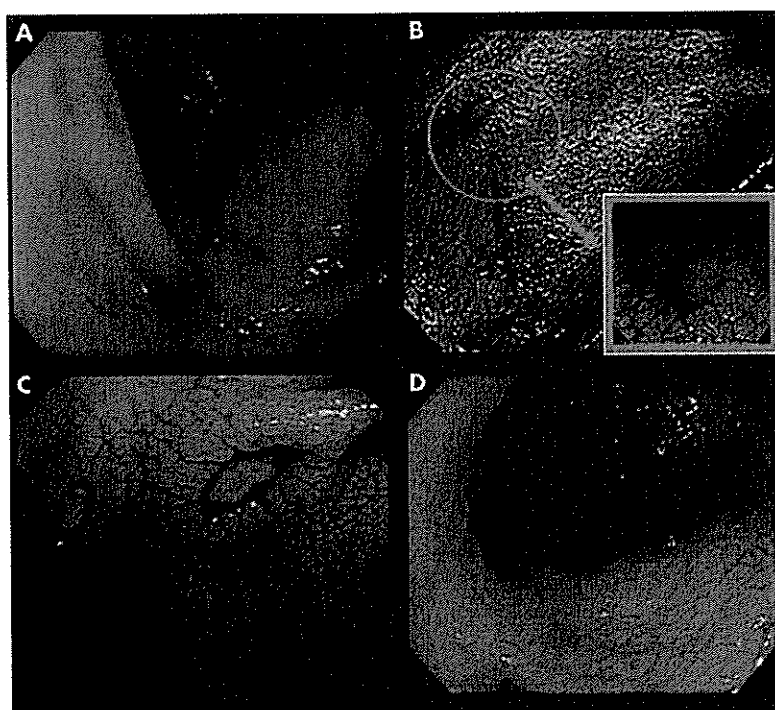


Figure 1 Endoscopic findings of cytomegalovirus (CMV) enterocolitis. (A) Erosion. (B) Erosion after indigo carmine dye spray. (C) Ulceration. (D) Oozing.

Table 1 Endoscopic findings and localisation of cytomegalovirus (CMV) inclusion bodies in patients with CMV enterocolitis

	Colonoscopic findings				Localisation of CMV inclusion bodies							CMV antigenaemia
	Erosion	Ulceration	Oozing	Other	T/I	Caecum	A/C	T/C	D/C	S/C	Rectum	
Case 1	NA	NA	1/3	0/2					1/2†	0/1	0/2	50
Case 2	1/2	NA	NA	0/5		1/2‡	0/1‡	0/1	0/1	0/1	0/1	14
Case 3	1/4	NA	NA	0/3	0/1	0/1	0/1	1/1‡	0/1‡	0/1‡	0/1‡	8
Case 4	NA	NA	1/5	NE						0/2	1/3	296
Case 5	NA	NA	NA	3/3*	1/1	1/1					1/1	61
Case 6	5/5	NA	NA	NE				3/3	2/2			932
Case 7	1/1	0/3†	NA	NE	0/1			1/4‡				871
Case 8	5/7	NA	NA	0/2	0/2	2/2‡	0/2	3/3‡				108

Each column represents total CMV inclusion bodies per biopsy specimens.

*Biopsy specimens were obtained from the edge of the exfoliation of the mucosa.

†Punched out ulceration.

‡Presence of erosion.

NA, not applicable; NE, not examined; T/I, terminal ileum; A/C, ascending colon; T/C, transverse colon; D/C, descending colon; S/C, sigmoid colon.

References

- Stocchi R, Ward KN, Fanin R, et al. Management of human cytomegalovirus infection and disease after allogeneic bone marrow transplantation. *Haematologica* 1999;84:71–9.
- Spencer GD, Hackman RC, McDonald GB, et al. A prospective study of unexplained nausea and vomiting after marrow transplantation. *Transplantation* 1986;42:602–7.
- Kanda Y, Mineishi S, Saito T, et al. Response-oriented preemptive therapy against cytomegalovirus disease with low-dose ganciclovir: a prospective evaluation. *Transplantation* 2002;73:568–72.
- Wilcox CM, Chalasani N, Lazenby A, et al. Cytomegalovirus colitis in acquired immunodeficiency syndrome: a clinical and endoscopic study. *Gastrointest Endosc* 1998;48:39–43.
- Hinnant KL, Rotterdam HZ, Bell ET, et al. Cytomegalovirus infection of the alimentary tract: a clinicopathological correlation. *Am J Gastroenterol* 1986;81:944–50.
- Iwasaki T. Alimentary tract lesions in cytomegalovirus infection. *Acta Pathol Jpn* 1987;37:549–65.
- Roberts WH, Sneddon JM, Waldman J, et al. Cytomegalovirus infection of gastrointestinal endothelium demonstrated by simultaneous nucleic acid hybridization and immunohistochemistry. *Arch Pathol Lab Med* 1989;113:461–4.

Hyperhomocysteinaemia and vascular disease in liver patients

We read with interest the case report by Buchel *et al* (*Gut* 2005;54:1021–3). The authors reported on a patient with portal hypertension, nodular regenerative hyperplasia, and portal thrombosis that presented an avascular hip necrosis after liver transplantation (OLT). The authors suggested that the whole clinical picture might stem from hyperhomocysteinaemia (high Hcy) and 677C→T heterozygosity for the common gene of methylene tetrahydrofolate reductase (MTHFR).

Although the history is suggestive of this hypothesis, high Hcy might also be a secondary phenomenon. It is not clear whether high Hcy was demonstrated in a sample obtained before (as suggested in table 1) or after (as suggested in the text) OLT. In both cases, Hcy might be elevated as a consequence of either liver disease or drug treatment. Liver disease per se raises Hcy due to the multiple metabolic problems generated by a failing liver, including low folate levels, altered transsulphuration/transmethylation pathway, and decreased renal function. High Hcy is present in 50% of liver disease patients,

and values as high as 30 µmol/l are not uncommon in end stage disease,¹ independently of MTHFR polymorphism. After OLT, a specific effect of tacrolimus on Hcy metabolism and concentration is well documented. Both calcineurin inhibitors (cyclosporin and tacrolimus) are known to interfere with the folate dependent remethylation of Hcy, thus raising plasma levels to values in the pathological range. The problem is well documented in patients following renal,² cardiac,³ or liver transplantation.⁴ In a recent analysis of 230 patients subjected to OLT, we found 26 cases with fasting Hcy ≥30 µmol/l in the late post-transplant phase, independently of MTHFR polymorphism.⁵

Also, the relationship between high Hcy and MTHFR polymorphism deserves comment. The MTHFR gene independently and unfavourably influences homocysteine metabolism but high Hcy levels are mainly observed in subjects with low folate levels,⁶ indicating a low phenotypic expression. Therefore, the presence of the genetic variant, mainly in its heterozygous form, might be an occasional phenomenon without clinical significance.

In conclusion, although the clinical history of the patients raises the suggested pathogenic role of Hcy and genetic predisposition, no clues can be made on the “egg and chicken” sequence, particularly in a patient with positive markers for hepatitis B infection.

Adequate folate intake or pharmacological supplementation may certainly overcome genetic predisposition to high Hcy, but may also decrease Hcy, independently of any gene defect, and remains a suitable therapeutic option to prevent additional vascular problems.

G Bianchi, G Marchesini, M Zoli

Department of Internal Medicine, “Alma Mater Studiorum” University of Bologna, Bologna, Italy

Correspondence to: Dr G Bianchi, Department of Internal Medicine, Cardioangiology, Hepatology, “Alma Mater Studiorum” University of Bologna, Policlinico S Orsola-Malpighi, Via Massarenè, 9, I-40138 Bologna, Italy; gjampolo.bianchi@unibo.it

Competing interest: None declared.

References

- Bosy-Westphal A, Ruschmeyer M, Czech N, et al. Determinants of hyperhomocysteinaemia in patients with chronic liver disease and after orthotopic liver transplantation. *Am J Clin Nutr* 2003;77:1269–77.

- Fernandez-Miranda C, Gomez P, Diaz-Rubio P, et al. Plasma homocysteine levels in renal transplanted patients on cyclosporine or tacrolimus therapy: effect of treatment with folic acid. *Clin Transplant* 2000;14:110–14.
- Cole DE, Ross HJ, Evrosvki J, et al. Correlation between total homocysteine and cyclosporine concentrations in cardiac transplant recipients. *Clin Chem* 1998;44:2307–12.
- Bianchi G, Nicolino F, Passerini G, et al. Plasma total homocysteine and cardiovascular risk in patients submitted to liver transplantation. *Liver Transplant* 2006;12:105–11.
- Potena L, Grigioni F, Viggiani M, et al. Interplay between methylenetetrahydrofolate reductase gene polymorphism 677C→T and serum folate levels in determining hyperhomocysteinaemia in heart transplant recipients. *J Heart Lung Transplant* 2001;20:1245–51.

Second gastric cancers among patients with primary sporadic and familial cancers in Sweden

Gastric cancer ranks as the fourth most common cancer and the second most frequent cause of cancer death worldwide.¹ The aetiology includes causes such as *Helicobacter pylori* infection (for distal gastric cancer but not for cardia cancer),² dietary imbalance, smoking, and genetic factors.^{2,3} Estimation of the incidence of second primary cancers may provide valuable insight into the aetiology and shared risk factors with the initial cancer. However, as gastric cancer patients have poor survival,⁴ a study of second malignancies after primary gastric cancer may not be informative. Instead, we examined the occurrence of second gastric cancers following any first cancers, based on the nationwide Swedish Family Cancer Database. This database has been described in detail previously.⁵ Briefly, it was created by linking information from the Multigenerational Register, censuses, Cancer Registry, and death notifications. The database has an almost complete follow up of registered cancer patients and it provides a unique opportunity to quantify the risks of developing second gastric cancers among all primary cancer patients. Person years at risk were accumulated for each subject from the data of diagnosis of the first malignancy to that of a second gastric cancer, death, emigration, or 31 December 2002, which came first. Standardised incidence ratio (SIR) was used to estimate the risks of second gastric cancers, adjusted for sex, age, period, residence, and socioeconomic level. Confidence intervals were calculated assuming a Poisson distribution. Family history included all first

Coincidental Outbreak of Methicillin-Resistant *Staphylococcus aureus* in a Hematopoietic Stem Cell Transplantation Unit

Osamu Imataki,¹ Atsushi Makimoto,^{1*} Shingo Kato,² Takahiro Bannai,³ Naomi Numa,⁴ Yoko Nukui,⁵ Yuji Morisawa,⁶ Toshihiko Ishida,⁷ Masahiro Kami,¹ Takahiro Fukuda,¹ Shin-ichiro Mori,¹ Ryuji Tanosaki,¹ and Yoichi Takaue¹

¹ Hematopoietic Stem Cell Transplantation Unit, National Cancer Center Hospital, Tokyo, Japan

² Department of Microbiology and Immunology Division, Keio University School of Medicine, Tokyo, Japan

³ Laboratory of Microbiology, National Cancer Center Hospital, Tokyo, Japan

⁴ Nursing Division, National Cancer Center Hospital, Tokyo, Japan

⁵ Department of Infectious Diseases, University of Tokyo Hospital, Tokyo, Japan

⁶ Department of Infection Control and Prevention, Jichi Medical School, Tochigi, Japan

⁷ First Department of Internal Medicine, Kagawa Medical University Hospital, Kagawa, Japan

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common nosocomial pathogens among hospital-acquired infections, and immunocompromised patients are highly susceptible to infection. The molecular typing of isolated strains is a common method for tracing an outbreak of MRSA, but experience with this approach is still limited in the hematopoietic stem cell transplantation (HSCT) ward.

Methods: We experienced 6 cases of MRSA infection/colonization in our 26-bed HSCT ward during a 4-week period. This unusual outbreak strongly suggested that the same MRSA strain was involved despite strict isolation and aseptic patient care. Clarification of the transmission pattern was critical, and we applied pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) assays for evaluation.

Results and conclusion: In four of the six cases, the pattern of bands examined by PFGE and AFLP analyses supported the idea that direct person-to-person transmission was very unlikely and the outbreak was coincidental. This experience highlights the clinical value of molecular typing methods for the clinical epidemiological assessment of MRSA outbreak. Am. J. Hematol. 81:664–669, 2006 © 2006 Wiley-Liss, Inc.

Key words: outbreak; MRSA; stem cell transplantation

INTRODUCTION

The rapid increase in the incidence of hospital-acquired infection by methicillin-resistant *Staphylococcus aureus* (MRSA) is making infection control procedures very critical, particularly for immunocompromised patients [1]. Hospital-acquired infections also serve as a hallmark of the effectiveness and quality of infection control maneuvers [2]. Outbreaks of infection caused by MRSA have time-consuming and expensive consequences, and genetic analysis is useful, since it can be used to determine the route and origin of MRSA infection [3]. Currently available laboratory methods for determining DNA fragment sizes or sequences in MRSA isolates include Southern blotting [4], ribotyping [5], polymerase chain reaction (PCR) [6], and pulsed-field gel

electrophoresis (PFGE) [4,7]. PFGE has become the most common tool for the rapid discrimination of MRSA strains due to its convenience, reliability, and cost-effectiveness [8,9]. However, the interpretation of PFGE bands still needs to be standardized [10]. Alternatively, the amplified fragment length

*Correspondence to: Atsushi Makimoto, Division of Pediatrics, National Cancer Center Hospital, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: amakimot@ncc.go.jp

Received for publication 12 September 2005; Accepted 24 February 2006

Published online 22 June 2006 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/ajh.20668

TABLE I. Clinical Characteristics of 6 Cases*

UNP	Age	Sex	Disease	Day after transplantation	Cause of admission	Admission to hospital	First admission ward	Admission to SCT unit
1	53	M	MDS	109	GVHD	2002/11/13	Ward A	11/15/2002
2	35	M	NHL	219	GVHD	2002/11/20	Ward B	11/27/2002
3	35	M	GCT	43	GVHD	2002/11/29	Ward C	12/5/2002
4	58	M	MDS	-13	SCT	2002/10/31	SCT unit	10/31/2002
5	54	F	NHL	-22	SCT	2002/11/5	SCT unit	11/5/2002
6	63	M	CML	210	GVHD	2002/11/5	SCT unit	11/5/2002

*Cases 4 to 6 were admitted before case 1 showed severe intestinal symptoms induced by gut GVHD, complicated with continuous gastrointestinal bleeding. In 4 of these 6 cases, hospitalization was due to GVHD after transplantation (3 acute and 1 chronic), and 3 of these patients, including case 1, received corticosteroid therapy for the treatment of GVHD.

Note: MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; GCT, germ cell tumor; CML, chronic myelogenous lymphoma; GVHD, graft versus host disease; PSL, prednisolone; SCT, stem cell transplantation.

polymorphism (AFLP) method is based on the selective amplification by PCR of a subset of restriction fragments from a digest of the whole bacterial genome [11,12]. AFLP has advantages over PFGE since it has more power for discriminating between different strains more quickly with higher specificity in the recognition of digestive fragments of whole bacterial genome [13].

The goal of these laboratory tests is to provide firm evidence that isolates, which are epidemiologically related during an outbreak of the infection, are also genetically related and thus represent the same strain. To enhance the reliability of such molecular laboratory results, the combined use of various genotyping methods appears to be effective [14,15]. An outbreak has been defined as infectious disease derived from the same pathogen, while an outbreak that originates from strains that are indistinguishable from each other by typing methods but for which no direct linkage can be demonstrated is called an "endemic outbreak" [16]. It has been suggested that in disease outbreak due to endemic strains, the common origin may be temporally distant from those in outbreak strains. From the perspective of infection control, this difference is critical, since different procedures are needed to prevent the spread of disease.

We experienced an outbreak of MRSA in our hematopoietic stem cell transplantation (SCT) ward that was initially suspected to be derived from a single origin. To address this serious problem, we tracked down the route of infection and obtained results that highlighted the clinical value of molecular typing using these methods.

PATIENTS AND METHODS

Patients

The routine infection-monitoring procedure in the SCT ward includes surveillance cultures and identifi-

cation for specific pathogens in the nasal swab, pharyngeal swab or sputum, urine, or stool, which are collected from patients who are suspected to have infection or colonization of the target pathogen including MRSA at the time of admission. In a 4-week period, we experienced six cases (UPN 1 to 6) of MRSA infection or colonization in the SCT ward, while the preceding incidence of MRSA detection in the SCT ward had been only one or two cases per month (mean 0.8/month, range 0-2/month, SD 0.61). Therefore, this was epidemiologically defined as an MRSA outbreak. The patient characteristics are summarized in Table I. We reviewed the medical records of the patients to collect the clinical information required to track down the transmission route. We documented the time course of MRSA identification in relation to patient characteristics, risks of nosocomial infection, and room assignment.

Samples

Isolates were grown from culturing sputum, urine, stool, pus, and blood, and a few were grown from culturing miscellaneous sites such as pharynx and nasal cavity. We examined the first sample isolated in each patient by molecular typing, PFGE, and AFLP analysis.

DNA Isolation and PFGE

Targeted bacterial strains were cultured at 37°C in Luria-Bertani broth. The cell component was lysed by proteinase K to extract DNA. Genomic DNA was digested with *Sma*I and resolved with the CHEF-DRII system (Bio-Rad Laboratories) as described by the manufacturer (traditional typing strategies) [17]. As a control strain, we used MRSA isolated from two groups: (1) two strains isolated from past patients in the same ward, which has no temporal relationship to our present cases (cases 7 and 8) and (2) five strains isolated from a different hospital

American Journal of Hematology DOI 10.1002/ajh

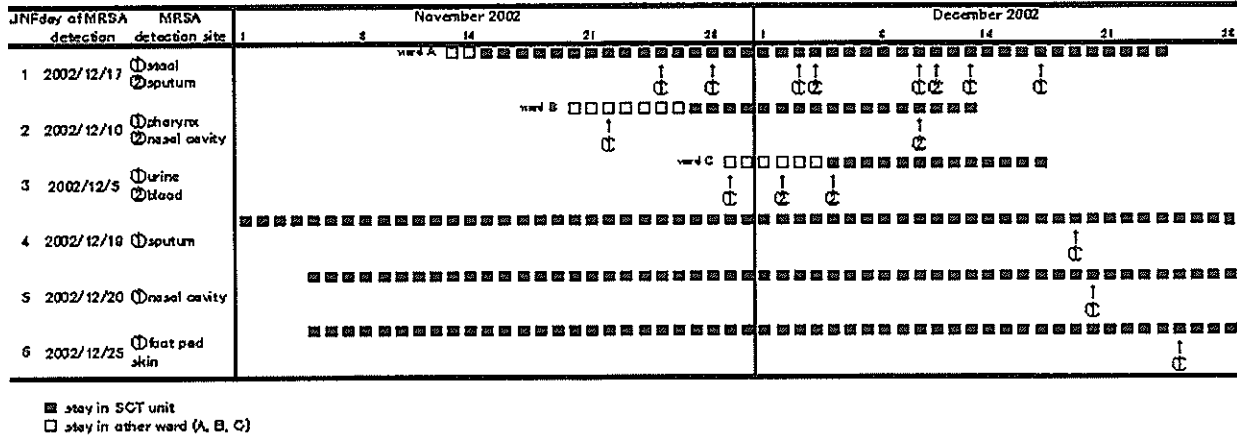


Fig. 1. Time course of MRSA detection in the SCT unit. MRSA was first isolated in the stool of case 1 on 25 November 2002. MRSA had been identified prior to admission to the SCT ward in cases 2 and 3. In contrast, in the other three cases (cases 4, 5, and 6), MRSA was detected after admission to the SCT ward.

(University of Tokyo Hospital, 1150 beds), which was not epidemiologically associated with our hospital (cases 9 to 13). PFGE banding was compared with that in case 1, who was thought to be the origin of this outbreak episode. The criteria described by Tenover et al. [16] were used for the molecular epidemiological interpretation of PFGE banding as follows:

- (i) indistinguishable: outbreak was derived from the same isolate;
- (ii) closely related: different isolates, closely related to the outbreak pattern;
- (iii) possibly related: different isolates, possibly related to the outbreak pattern;
- (iv) unrelated: different isolates, unrelated to the outbreak pattern.

One genetic event detected by PFGE was considered meaningful enough as different isolates.

AFLP

Bacterial DNA was prepared with a QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s recommendations. DNA was then manipulated with an AFLP Microbial Fingerprinting kit (Applied Biosystems) according to the manufacturer’s instructions based on a previous study [11]. Briefly, DNA was digested with *EcoRI* and *MseI* and then ligated to the corresponding adapters. This was followed by preselective amplification and selective amplification, where *EcoRI*-A (FAM), *EcoRI*-C (NED), *EcoRI*-G (JOE), and *MseI*-C primers were used. The AFLP reactions were evaluated by analyzing data from samples loaded and run on an ABI 310 Genetic Analyzer with GeneScan software. A dendrogram was constructed from a pairwise dis-

American Journal of Hematology DOI 10.1002/ajh

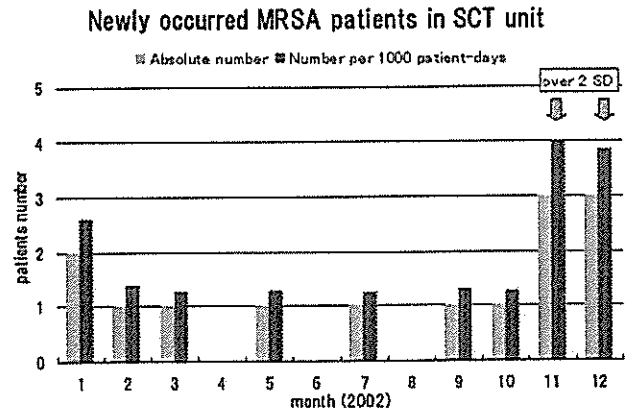


Fig. 2. The incidences of newly detected MRSA cases in SCT unit in 2002. Each bar indicates a number of patients clinically identified as MRSA in 2002.

tance matrix with the Clustal W version 1.8 software package.

Definitions [18]

“Methicillin-resistant” is defined according to NCCLS MIC criteria by dilution susceptibility tests. An “outbreak” of MRSA is defined as an increase in the rate of MRSA cases or a clustering of new cases in a specific place during a given period. In this report, we defined an unusual increase in MRSA cases as a multiply repeated isolation of MRSA from a physically independent ward (transplantation unit) with an incidence ≥ 2 SD over the baseline. The SCT unit is geographically separate from other wards and has an independent space that is managed to maintain sterilization. Patients from whom MRSA was isolated and who had any concomitant symptoms in the MRSA-detected part were referred to as “MRSA