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‡	—	1.25 (0.85-1.84)	.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.

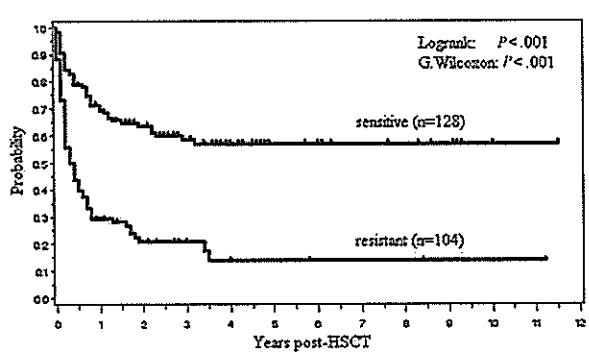

Figure 5. The relation between overall survival and response to chemotherapy.

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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

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

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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.

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 leukemia[9] 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 form Beckman Coulter (Fullerton, CA), and

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APC-conjugated CMV, WT1 and PRAME-tetramer were purchased from ProImmune (Oxford, UK). The antibodies and tetramer complexes were added to 200 μL 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 10^5) 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 1×10^6 cells/mL in RPMI 1640 (Sigma) supplemented with 10% FBS, gentamicin and streptomycin (hereafter referred to as culture medium). PBMC (2×10^6 cells) were seeded in a 24-well plate, and the peptide was added to a final concentration of 5 μM 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 μM peptide. Cells were cultured for 14 days.

Intracellular cytokine staining

The cultured cells were washed with culture medium, and 5×10^4 cells per test were suspended in 200 μL 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 μM and incubated for 6 hr at 37°C. Breferdin A (Sigma; 10 μg/mL) was added during the last 4 hr of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μg/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 1 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 A*0206, 1 had A*0207 and 1 had both A*0201 and A*0206 genotypes.

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% ≤ 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)*

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

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 GHVD 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

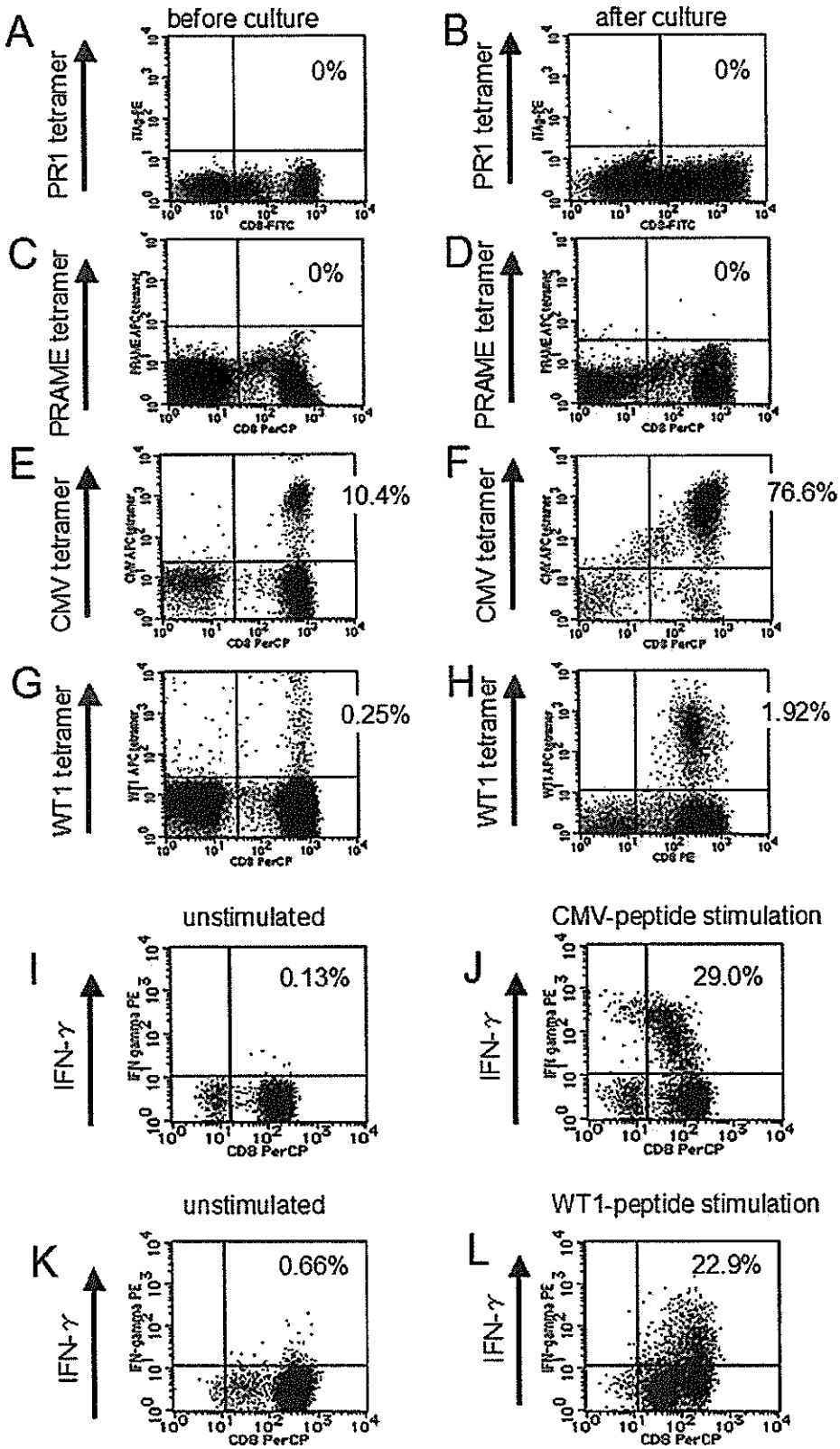

FIGURE 1 – Cell expansion of antigen-specific CTL and intracellular IFN-γ staining. PR1-CTL culture in CML patient (*a*, *b*) and PRAME-CTL culture in a RCC patient (*c*, *d*) remained in undetectable levels, even after expansion culture. CTL showed an expansion of CMV-CTL (*e*, *f*) and WT1-CTL (*g*, *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.

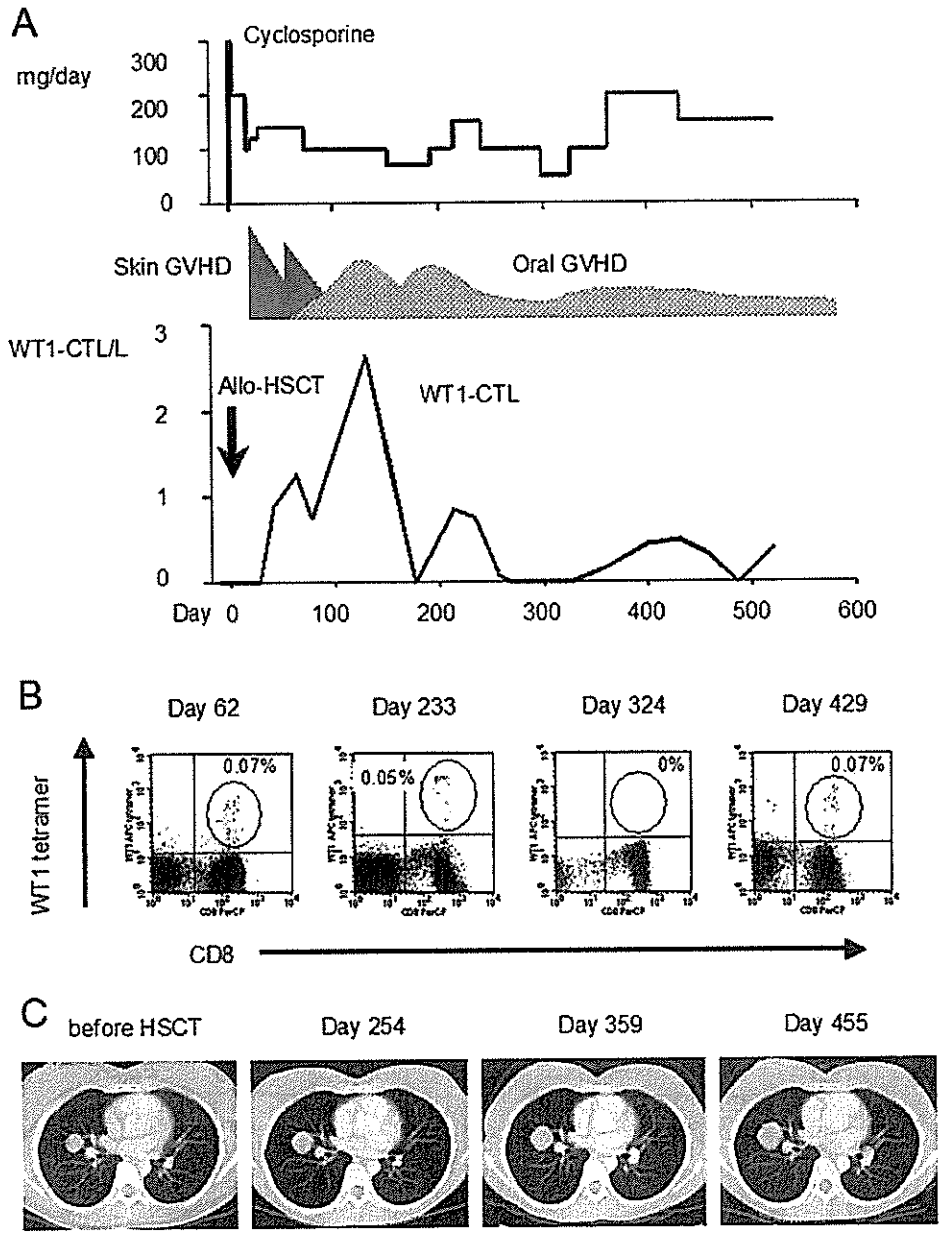

FIGURE 2 – Clinical course and tetramer analysis of Case 1 with RCC WT1-CTL was detected after the occurrence of skin GVHD, followed by a peak on day 128 when oral GVHD developed (*a*, *b*). Lung metastases slowly progressed while WT1-CTL disappeared (*c*).

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*–1*h*).

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-$tetramer^+$ lymphocyte 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 administrated as GVHD prophylaxis. Engraftment was achieved on day 12, which was followed by skin GVHD that extended to the whole

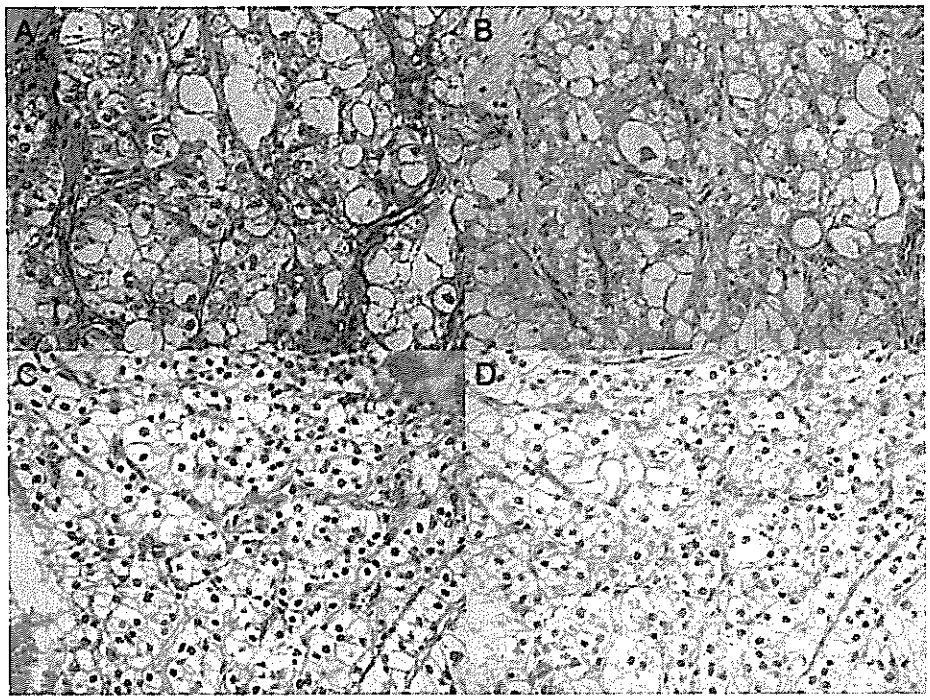

FIGURE 3 – Histology of resected RCC. The resected tumor sample in the first case with hematoxylin–eosin staining (*a*) confirmed a mixed cell carcinoma of the kidney. Immunostaining with WT1 (*b*) was positive for WT1. The second case with clear cell carcinoma (*c*) also showed positive staining for WT1 (*d*). Original magnification ×200 for (*a*, *b*) and ×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. 3*c* and 3*d*). 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 $CD45RA^+/CD45R0^-$ phenotype, 53% were of $CD57^+/CD45R0^-$ phenotype and 22% were of $CD57^-/CD45R0^-$ 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 $CD45RA^-/CD45R0^+$ 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 leukemia[25] 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.

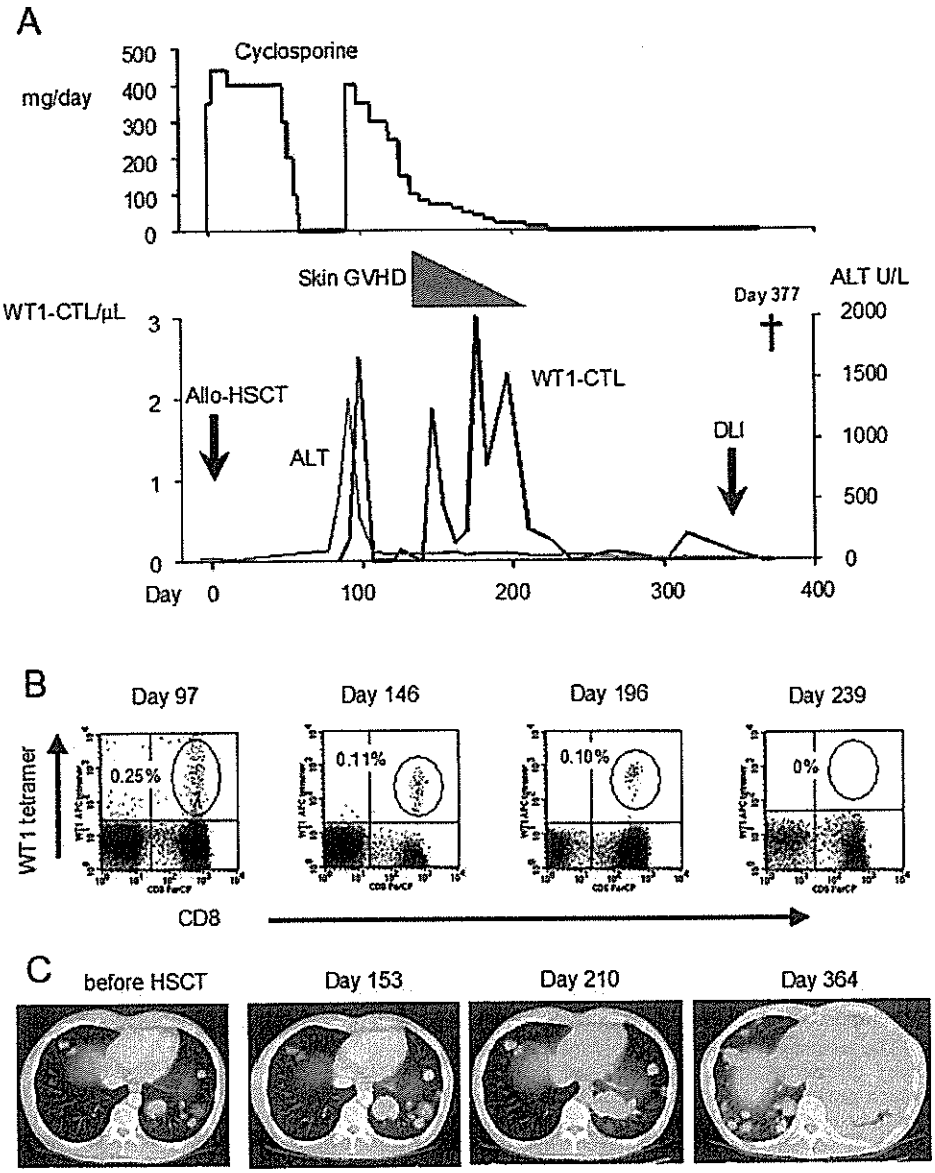

FIGURE 4 – Clinical course and tetramer kinetics in Case 2 with RCC. WT1-CTL emerged after the occurrence of liver and skin GVHD (*a*, *b*). The tumor rapidly grew after the disappearance of WT1-CTL (*c*).

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-

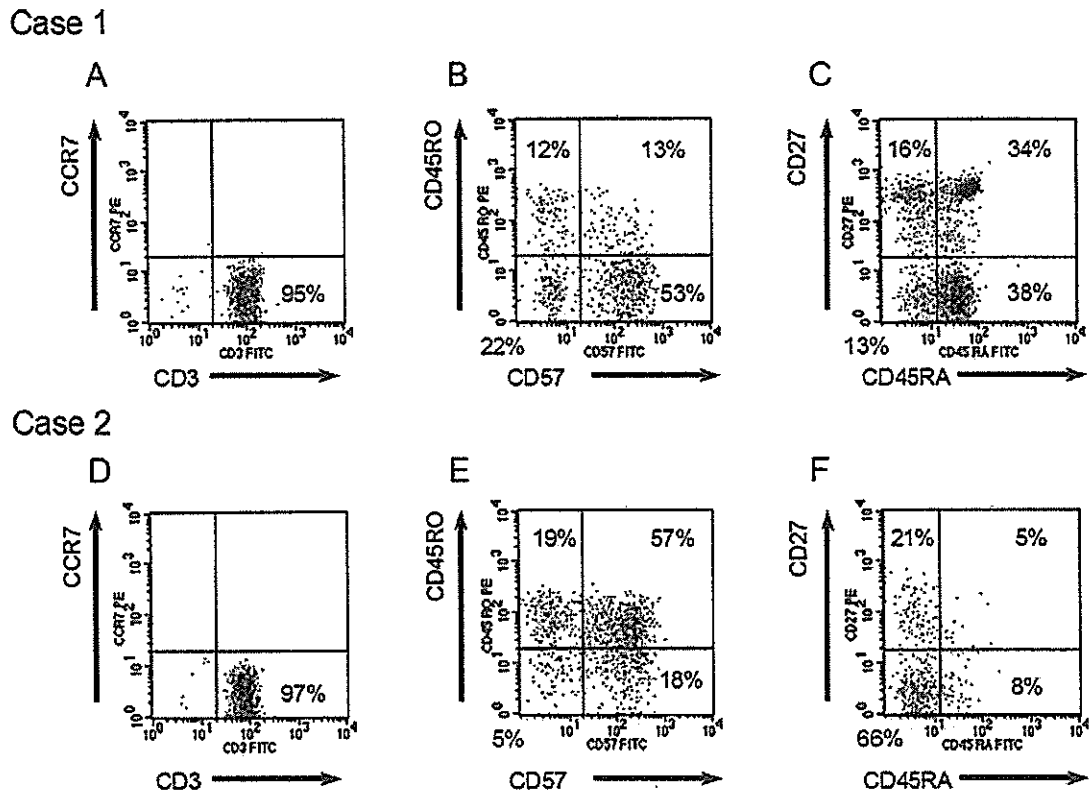

FIGURE 5 – Immunophenotype analysis of WT1-specific CTL. The immunophenotype of WT1-CTL in the first case of RCC was $CCR7^-$ and mainly $CD57^+/CD45RO^-/CDRA^+$ (*a–c*). The immunophenotype of WT1-CTL in the second case was $CCR7^-$ and $CD57^+/CD45RO^+$ and $CDRA^-/CD27^-$ (*d–f*).

notypes, 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. Monitoring 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.

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Endoscopic evaluation for cytomegalovirus enterocolitis after allogeneic haematopoietic stem cell transplantation

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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.

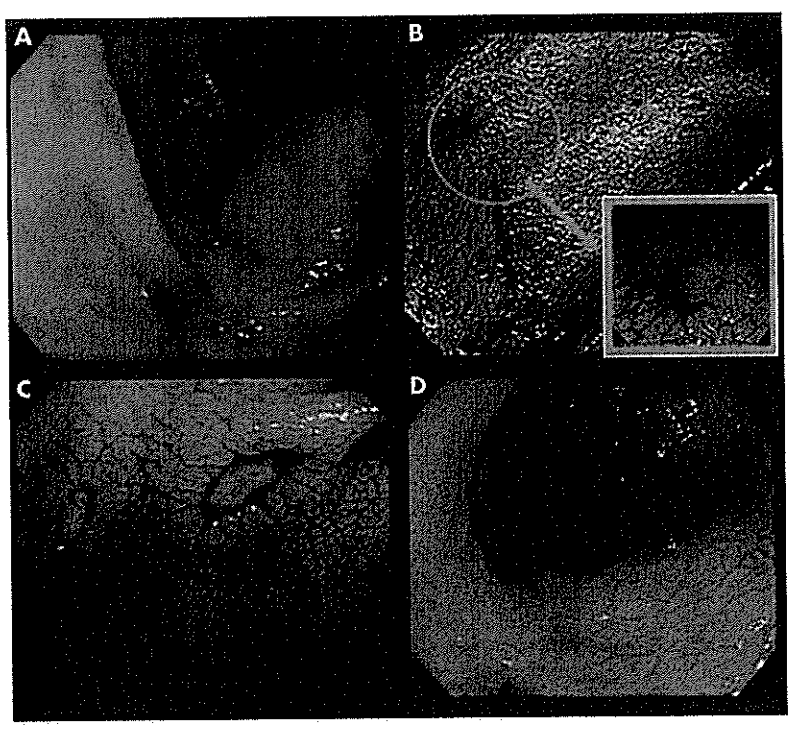

Figure 1 Endoscopic findings of cytomegalovirus (CMV) enterocolitis. (A) Erosion. (B) Erosion (after indigo carmine dye spray). (C) Ulceration. (D) Oozing.

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Conflict of interest: None declared.

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.

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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 (ciclosporin 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.[1] 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.[4]

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,[5] 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.

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Competing interest: None declared.

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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

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

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Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common nosocomial pathogens among hospital-acquired infections, and immunocompromised patients are highly susceptive 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

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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 myelogeneous 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 identification 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

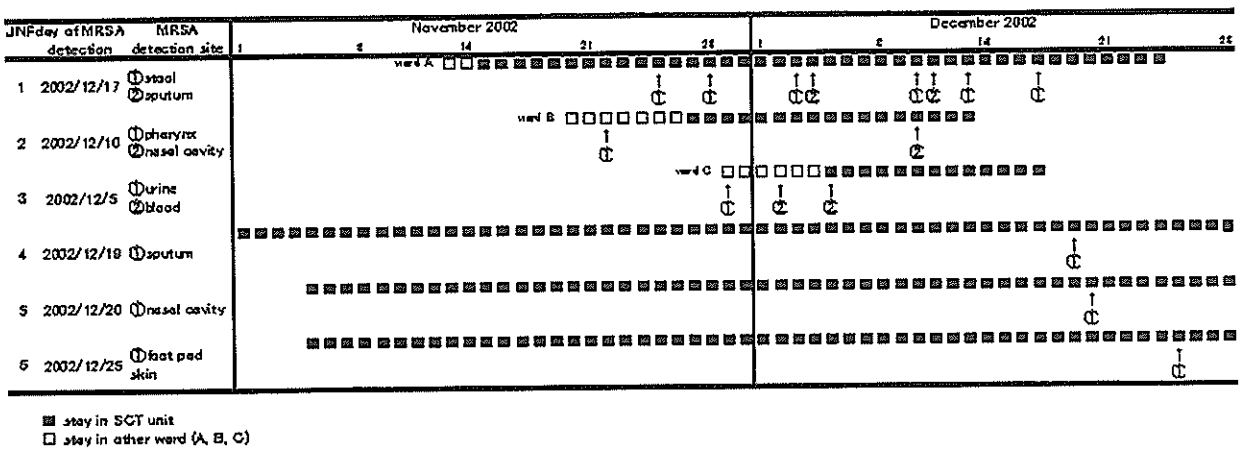

Fig. 1. Time course of MRSA detection in the SCT unit. MRSA was first isolated in the stool of case 1 on 25 November 2002. MRSA had been identified prior to admission to the SCT ward in cases 2 and 3. In contrast, in the other three cases (cases 4, 5, and 6), MRSA was detected after admission to the 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 *Eco*RI and *Mse*I and then ligated to the corresponding adapters. This was followed by preselective amplification and selective amplification, where *Eco*RI-A (FAM), *Eco*RI-C (NED), *Eco*RI-G (JOE), and *Mse*I-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 distance matrix with the Clustal W version 1.8 software package.

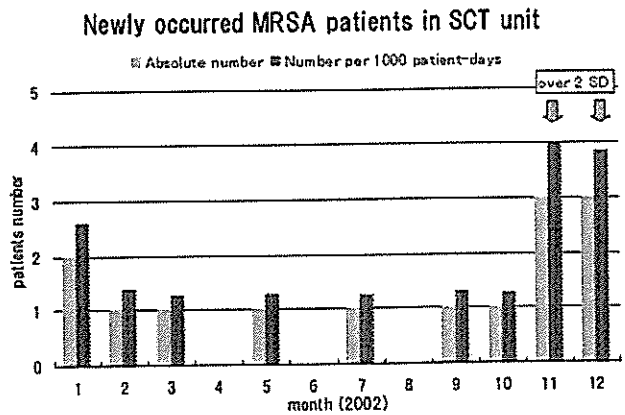

Fig. 2. The incidences of newly detected MRSA cases in SCT unit in 2002. Each bar indicates a number of patients clinically identified as MRSA in 2002.

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 $\geq$ 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

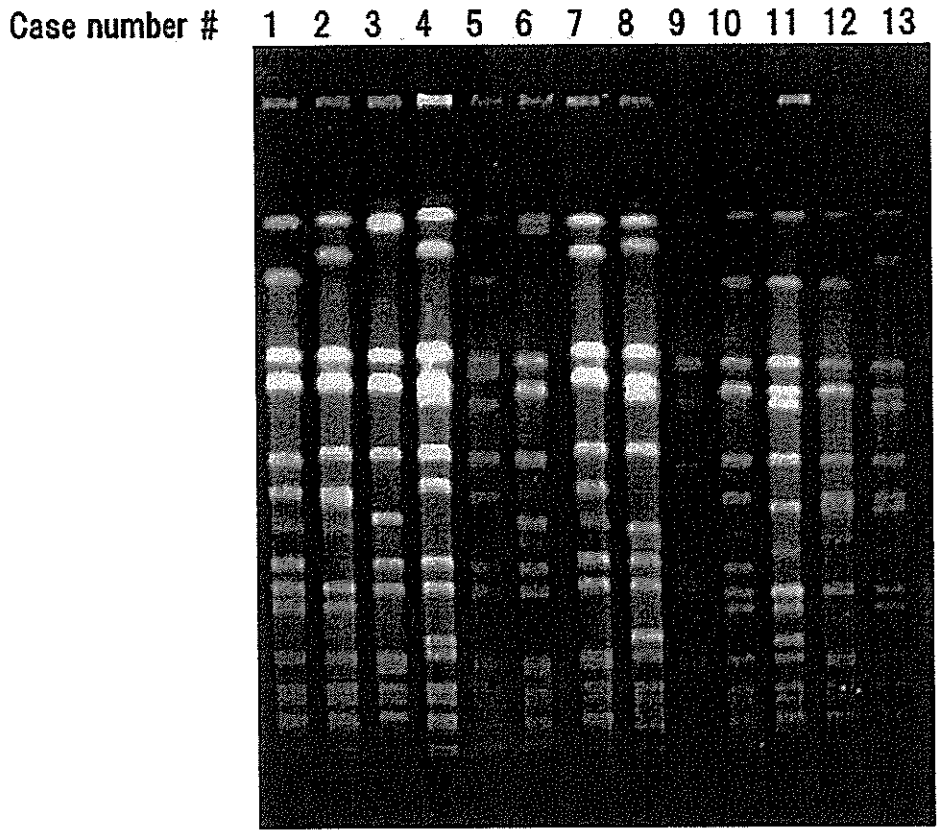

Fig. 3. PFGE analysis of isolated MRSA in the SCT unit. The PFGE pattern showed that there were no detectable differences in bands between cases 3 and 6, but more than two bands were identified in the other four cases (cases 1, 2, 4 and 5). Additionally, two strains that had been previously isolated in the SCT ward (cases 7 and 8) were distinguishable, and the five epidemiologically different isolates (cases 9 to 13) from the University of Tokyo Hospital (1,150 beds) were also distinguishable, with differences in more than two bands.

infection," while those without symptoms were considered "MRSA colonization."

RESULTS

Clinical Course of MRSA Outbreak

The clinical characteristics of six patients in whom MRSA was isolated are presented in Table I. The first patient (case 1) was admitted to the SCT ward because of severe intestinal symptoms induced by gut GVHD, chronic diarrhea, and continuous gastrointestinal bleeding, which occurred at 107 days after SCT. The patient received corticosteroid and intravenous antimicrobial therapy. For 4 weeks prior to his admission, there had been no case of MRSA infection or colonization in the ward. At 13 days after admission, the first isolation of MRSA in his stool was recorded (Table I). Subsequently, five other patients newly developed MRSA events over the next 4 weeks (Figure 1), while the incidence of MRSA detection of SCT ward had remained at one or two cases per month (mean 0.8 /month, range 0–2 /month, SD 0.61, Figure 2). Among these five cases, three (cases 4, 5, and 6) had been admitted to the SCT ward directly from the outpatient clinic without a past history of MRSA infection. The other two cases (cases 2 and 3) were transferred from other wards after the admission of case 1, and MRSA was isolated prior to transfer to the SCT ward (Figure 1). Since, in these two cases, MRSA was identified again in different site in SCT with different drug-sensitivity profile (data not shown) from a previous strain, we included these two cases in the analysis. There were no other patients who were previously identified with MRSA infection or colonization.

Tracing Procedure

The transmission, if any, appeared to take a random pattern, as illustrated in Figure 1. To better evaluate whether the transmission pattern was direct or indirect, we identified the layout of the patients' bed assignments. This revealed that there were nei-

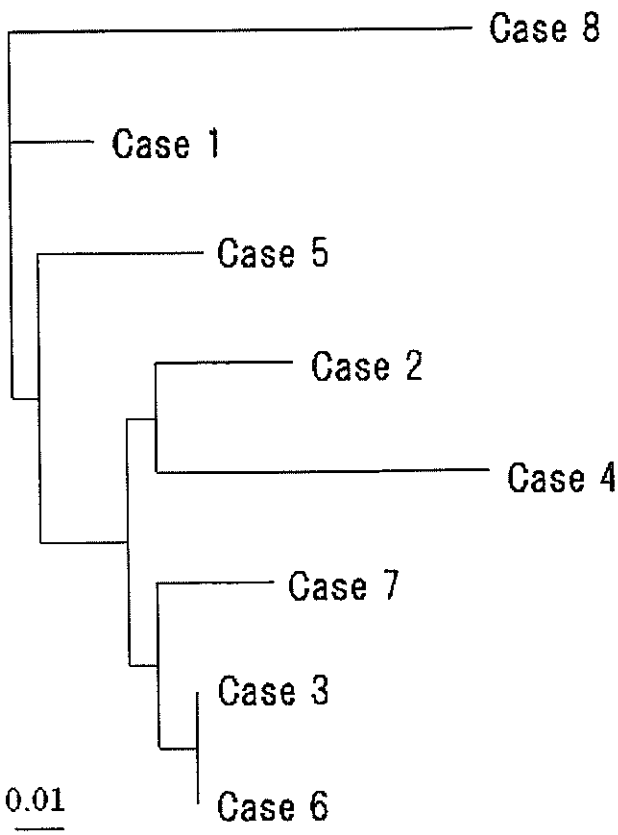

Fig. 4. AFLP pattern of isolated MRSA. AFLP analysis was performed for the same six isolates (case 1 to 6) described in Figure 4. Gene polymorphism showed same result of similarity as PFGE pattern had already indicated, i.e., cases 3 and 6 had the same polymorphism pattern and the others were different strains. The mutual relation of gene polymorphism is presented in the dendrogram and relatedness is indicated by the length of line. The scale bar drawn in the lower part indicated 1.0 % relatedness.

ther overlaps nor coexistence with preceding patients, except that cases 1 and 3 used the same room on different days without an overlap.

PFGE and AFLP Assays of MRSA Isolates

PFGE analysis of the six MRSA strains isolated (Figure 3) showed that two strains (cases 3 and 6) were indistinguishable and therefore considered to be derived from the same isolate, while the remaining four cases (cases 1, 2, 4, and 5) were considered to have different strains. Seven epidemiologically different isolates, i.e., two strains isolated from another ward at different times (cases 7 and 8) and five strains isolated from another hospital (cases 9 to 13) were used as in-hospital and extra-hospital controls, respectively. The results were confirmed by AFLP analyses as a dendrogram shown in Figure 4.

DISCUSSION

The spread of MRSA in highly protected care units, including ICU [19] and neonatal ICU [20], is a well-known major complication in compromised patients. Although few reports have been published on the outbreak of MRSA in a SCT unit, a continuous rise in the incidence of hospital-acquired MRSA infection [21] should influence the incidence of MRSA infection in SCT recipients [22]. Collin et al. reported that the incidence of multidrug resistant *S. aureus* was 15% in isolates from BMT patients with blood stream infection in 1991–1997 [23]. Prolonged neutropenia has been found to be a risk factor for the development of infectious complications in SCT recipients [24]. Since the outbreak of MRSA among immunocompromised patients can greatly affect their mortality, appropriate methods for infection control are strongly warranted. The Consensus Panel's guidelines for preventing the spread of MRSA recommend contact precautions and the isolation of infected or colonized patients in a single room or cohort, i.e., grouping them geographically with designated staff [18]. Also, since MRSA colonization precedes infection because of inpatient circumstances and rather strong treatments [25,26], a local control is very important for controlling MRSA outbreak in selected circumstances such as SCT ward in which many immunocompromised patients are taken care of.

In this report, we described an MRSA outbreak in the SCT ward during a limited period of 4 weeks. Initially, we suspected that all MRSA infections were caused by a single source, such as highly contaminated stool. However, unexpectedly, no direct contact was identified among patients and staff who were involved in their care. The transmission of MRSA mostly occurs through direct person-to-person contact, and transmission from the environment is extremely rare in places where strict precautions are taken and careful decontamination procedures are used. Hence, we undertook a molecular epidemiological analysis to critically examine the suspected break in our procedure. We found that four of the six isolates were genetically different, and our Infection Control Team concluded that horizontal transmission was unlikely. Nevertheless, the interest raised with this event resulted in further enforcement of essential precautions against droplets and contact,and the elimination of new MRSA cases for subsequent months.

Although our observation was well anticipated, in that molecular typing techniques are effective in the diagnosis and tracking of MRSA, the results are still unique, since they highlight the value of these methods over clinical judgment in a critical care situation

with highly immunocompromised patients. Since the molecular typing properties of MRSA are very similar in Japan, especially in the local areas [27], we focused on the genetic event detected by molecular typing and diagnosed those differences as different strain from outbreak. Thus, this report should be helpful for evaluating whether the routine application of these measures should be critically considered in the assessment of outbreak.

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American Journal of Hematology 81:747–752 (2006)

Nutritional Support for Patients Suffering From Intestinal Graft-versus-Host Disease After Allogeneic Hematopoietic Stem Cell Transplantation

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Background: Patients who exhibit gastrointestinal (GI) involvement due to graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (SCT) are often recommended to withhold oral intake (NPO) to avoid further damage to the GI mucosa. However, it is possible that continuing oral intake could be beneficial in many patients compared to total parenteral nutrition (TPN).

Objective: The primary objective of this prospective study was to evaluate whether programmed step-ladder oral dieting (enteral nutrition; EN) is feasible and beneficial for these patients.

Methods: A total of 18 patients who exhibited GI-acute GVHD (stage I to III gut GVHD) after SCT received an EN dieting program, and changes in clinical and laboratory parameters were compared to those in a control cohort of 17 patients who were placed on NPO with TPN. Patients with GVHD were included prospectively and those with intestinal bleeding/obstruction, severe pancreatitis, and cytomegalovirus enterocolitis were excluded.

Results: None of the patients in the EN group experienced significant adverse events, including exacerbation of GI symptoms. Although there was no statistically significant difference in the volume or frequency of diarrhea or the time to complete dietary recovery, parameters including body weight and serum levels of total protein and albumin tended to improve faster in the EN group.

Conclusion: The EN diet is safely applicable to patients suffering from GI involvement by GVHD. Am. J. Hematol. 81:747–752, 2006. © 2006 Wiley-Liss, Inc.

Key words: graft-versus-host disease (GVHD); enteral nutrition; immunonutrition

INTRODUCTION

Graft-versus-host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation (SCT) that influences the ultimate prognosis of patients [1]. Gut involvement due to GVHD particularly impairs the host nutritional status and QOL due to long-lasting diarrhea and anorexia. Hence, effective supportive care of patients suffering from GVHD should include attention to intense nutritional support and bone mineral retention, since many receive concomitant steroid therapy. Additionally, normal intestinal architecture and functions are required to prevent biliary stasis, retarded bowel movement, bacterial translocation, and resultant systemic infection [2,3]. With the development of gut GVHD, patients are often recommended to withhold oral intake (NPO, "bowel rest") to avoid further damage to the gastrointestinal (GI) mucosa. However, this raises a serious concern since NPO care can induce atrophic deficit of the GI mucosa and resultant dysfunction

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