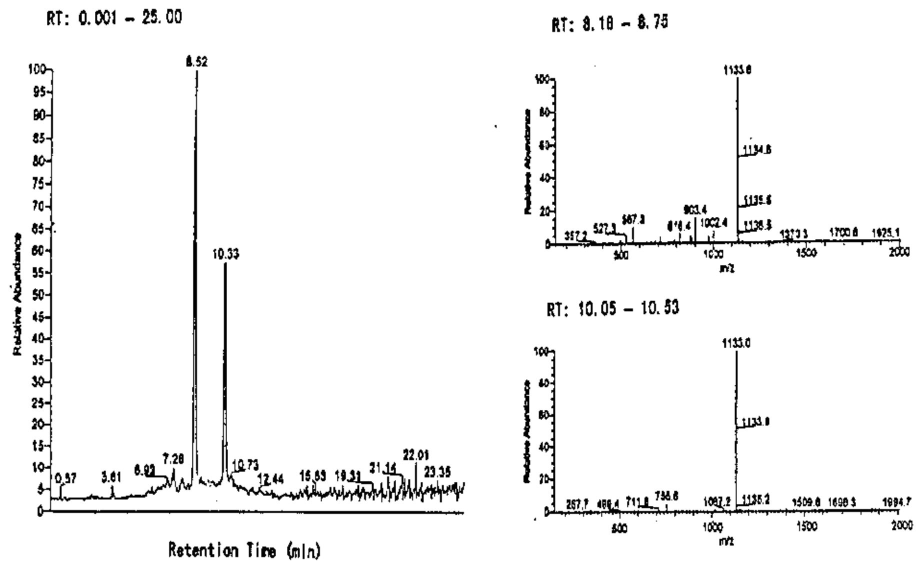

FIGURE 7. Both monomeric and dimeric forms of the $UBE2V_{43-51}$ peptide are shown. Chromatography and mass spectrometric analysis were conducted on the vaccinated $UBE2V_{43-51}$ peptide. Two peaks, with retention times of ~8.52 and ~10.33, were observed by chromatography. With regard to the mass spectrometric analysis, the peak at retention time ~8.52 had $(M+H)^+ = 1133.6$ and $(M + 2H)^{2+}/2 = 567.3$. The peak at retention time ~10.33 had $(M2 + 2H)^{2+}/2 = 1133.0$ and $(M2 + 3H)^{3+}/3 = 755.6$, and the presence of the latter fragment indicated that the peak at the later retention time (~10.33) was a dimer. RT, retention time.

unknown, it may be that the dimeric peptides lie in parallel on HLA-DR molecules. Thus, recognition of peptide-class II complexes by $CD4^+$ T cells might be not as rigid as recognition of peptide-class I complexes by $CD8^+$ T cells.

Although it was important to determine whether $UBE2V_{43-51}$ peptide-recognizing $CD4^+$ T cells or UBE2V peptide-reactive IgG can respond to tumor cells, HLA-DRB1*0403-expressing epithelial cancer cell lines were not available. Therefore, we determined whether the $UBE2V_{43-51}$ peptide-recognizing $CD4^+$ T cells could recognize autologous dendritic cells, which were pulsed with lysates from panc-1 cells, from which the *UBE2V* gene was cloned (6). However, no response was observed (data not shown). In regard to Ab, we attempted to determine, by two different methods, whether the IgG contained in the postvaccination serum of this patient could bind to the $UBE2V_{43-51}$ peptide-pulsed HM-LCL cells. In flow cytometric analysis, the HLA-DRB1*0403-expressing HM-LCL cells, which were prepulsed with the $UBE2V_{43-51}$ peptide, then cultured with postvaccination serum, followed by anti-human IgG second Ab, were not positively stained (data not shown). In the cytotoxicity assay, the $UBE2V_{43-51}$ peptide-pulsed HM-LCL cells were not killed by freshly isolated PBMCs in the presence of the heat-inactivated serum, suggesting the absence of Ab-dependent cell-mediated cytotoxicity (data not shown). We further investigated the possibility that vaccine-induced IgG reactive to the $UBE2V_{43-51}$ peptide merely recognized cysteine-based modification of the UBE2V peptide. We determined whether substitution of the sixth cysteine for alanine or serine could have any effect on recognition by IgG, and we observed no remarkable change in Ab titers against these two modified UBE2V peptides (data not shown). This indicates that the $UBE2V_{43-51}$ peptide-reactive IgG did not recognize cysteine-based modification of the $UBE2V_{43-51}$ peptide. We obtained no evidence that anti-$UBE2V_{43-51}$ peptide IgG was tumor reactive. We speculate that the induction of IgG reactive to the $UBE2V_{43-51}$ peptide was

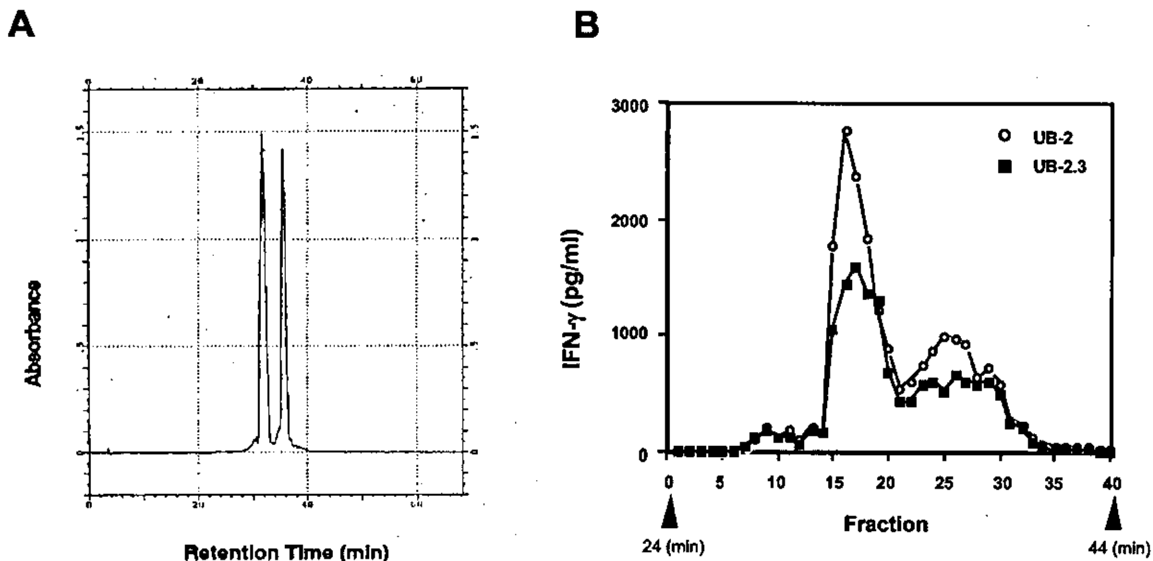

FIGURE 8. Recognition of fractionated $UBE2V_{43-51}$ peptide by UB-2 and UB-2.3 cells. *A*, Reverse phase HPLC was conducted on the vaccinated $UBE2V_{43-51}$ peptide. *B*, Forty fractions from 24–44 min in reverse phase HPLC were suspended by 50 μl of cysteine-free DMEM, and each 10 μl was separately pulsed on the HM-LCL cells. Thereafter, UB-2 or UB-2.3 cells were added and cultured overnight. The level of IFN-γ in the supernatant was determined by ELISA.

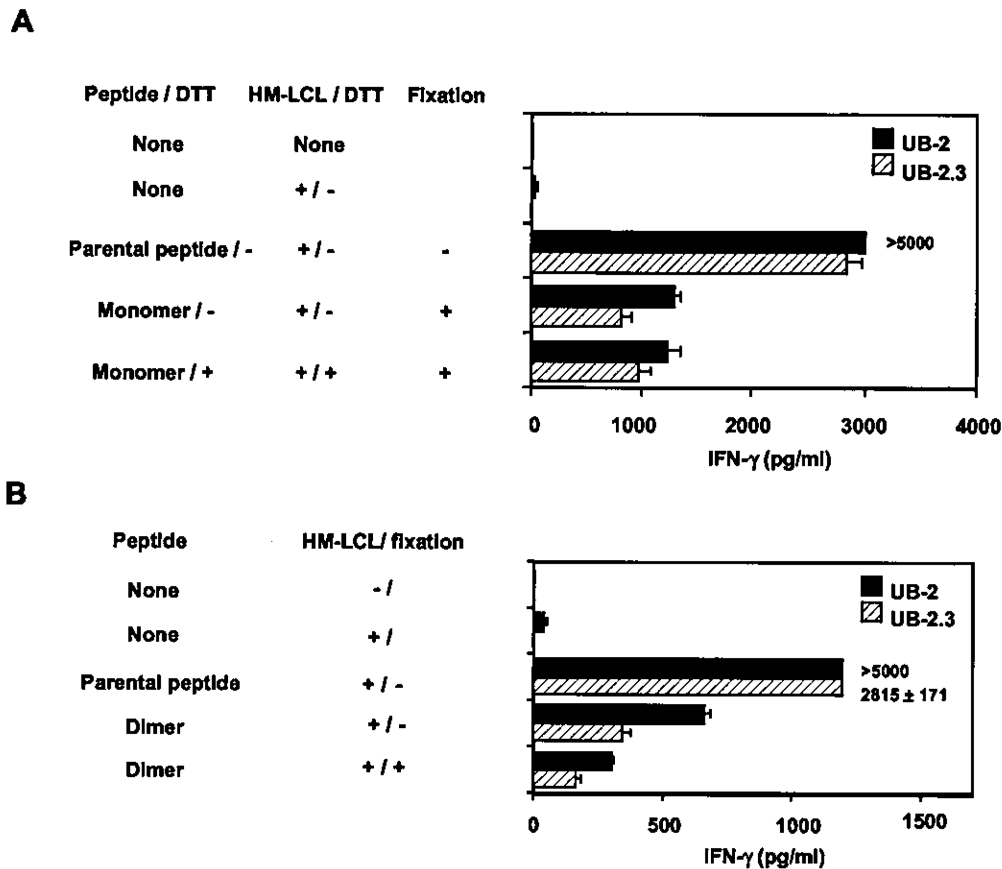

FIGURE 9. Recognition of both monomeric and dimeric $UBE2V_{43-51}$ peptide by UB-2 and UB-2.3 cells. *A*, The monomeric $UBE2V_{43-51}$ peptide (fractions 15–17 in Fig. 8*B*) and the HM-LCL cells were incubated with or without DTT (2 mM) for 2 h. Thereafter, the peptide and HM-LCL cells were mixed and cultured for an additional 90 min. After washing the cells, peptide-pulsed HM-LCL cells were fixed with 1% paraformaldehyde for 5 min. These treated HM-LCL cells were cultured with UB-2 or UB-2.3 cells in cysteine-free DMEM with 2% human serum for 1 day. The level of IFN-γ in the supernatant was determined by ELISA. *B*, The dimeric $UBE2V_{43-51}$ peptide (fractions 25–27 in Fig. 8*B*) was pulsed on the prefixed HM-LCL cells. After 90-min incubation, the cells were cultured with UB-2 or UB-2.3 cells in cysteine-free DMEM with 2% human serum for 1 day. The level of IFN-γ in the supernatant was determined by ELISA.

occurred because the UBE2V peptide is a good epitope not only for B cells, but also for $CD4^+$ T cells.

In most protocols involving peptide-based vaccines, the induction of peptide-specific CTLs has been examined after vaccination, without consideration of whether the peptide-specific CTL precursors were pre-existing in cancer patients. We have recently conducted clinical trials in which cancer patients were vaccinated with peptides (maximum of four) to which pre-existing CTL precursors in the periphery were confirmed before vaccination (22, 23). In this protocol the profiles of the vaccinated peptides varied among patients, and the $UBE2V_{43-51}$ peptide was vaccinated into three $HLA\text{-}A2^+$ cancer patients (one with thyroid cancer, one with mastocarcinoma, and one with seminoma) as one of four peptides (22). As a result, anti-$UBE2V_{43-51}$ peptide IgG was induced in all three patients after the third vaccination. This means that the dramatic induction of IgG reactive to administered peptides is not limited to the present case. In addition, peptide vaccination resulted in the induction of IgG reactive to several tumor peptides other than the $UBE2V_{43-51}$ peptide (22, 23). This indicates that the elicitation of IgG reactive to administered peptides is not limited to the $UBE2V_{43-51}$ peptide. In addition, we found that vaccine-induced, peptide-specific IgG tended to detectable in advanced lung cancer patients with a long progression-free survival (23). Although many clinical trials of peptide-based immunotherapy have been conducted to induce $CD8^+$ T cell-mediated tumor regression, no apparent correlation has been observed between in vivo tumor regression and the induction of peptide-specific CTLs in the periphery (3, 24). Our findings imply that the participation of class II-restricted $CD4^+$ Th cells should be considered in immunotherapy with class I binding tumor peptides, and that kinetic measurement of peptide-specific IgG could help to understand immune responses in vaccinated patients.

It is important to kinetically estimate the frequency of $CD8^+$ T cell or $CD4^+$ T cell precursors reactive to the $UBE2V_{43-51}$ peptide after the peptide vaccination. We estimated the frequency of peptide-specific T cells. The results showed that HLA-A2-restricted $CD8^+$ T cell precursors against the $UBE2V_{43-51}$ peptide were present in the prevaccination PBMCs, but their frequency increased after the 6th and 12th peptide vaccinations. In contrast, HLA-DRB1*0403-restricted $CD4^+$ T cell precursors against the $UBE2V_{43-51}$ peptide were detected only after the peptide vaccination. In our clinical trial, vaccination with four peptides, including the $UBE2V_{43-51}$ peptide, did not increase but rather abolished the pre-existing CTL precursors in the periphery in three $HLA\text{-}A2^+$ cancer patients (22), although the peptide vaccination induced $UBE2V_{43-51}$ peptide-specific IgG in these three patients. We have no explanation for the abolishment of peptide-specific CTL precursors in these patients, but we speculate that the concomitant induction of Th cells contributed to the increase in HLA-A2-restricted T cell response to the UBE2V peptide, at least in this particular patient.

The dominant HLA-A2 subtype in Caucasians is HLA-A*0201, whereas there is no dominant HLA-A2 subtype in Asians (25). The above-described patient was positive for HLA-A*0201 molecules. The UBE2V peptide was originally identified using HLA-A2-restricted and tumor-reactive CTLs from a colon patient (HLA-A*0207/3101) by cDNA library screening (6). The UBE2V peptide was prepared based on the binding motif to the HLA-A*0201 molecules. This CTL line produced IFN-γ in response to the T2 cells (HLA-A*0201) that were prepulsed with the UBE2V peptide and showed cytotoxicity against carcinoma cells, which express either HLA-A*0201 or HLA-A*0207 molecules (6). These results indicate that the $UBE2V_{43-51}$ peptide could be presented by several HLA-A2 subtypes.

There remains the question of why vaccination with the $UBE2V_{43-51}$ peptide induced DR4-restricted $CD4^+$ T cells in the patient. We propose the following explanations. First, the amino acid sequence of this peptide just matched the binding motif to HLA-DRB1*0403 molecules. Alternatively, the cysteine-based modification may have made the $UBE2V_{43-51}$ peptide resistant to degeneration by protease, and the peptide may have maintained its immunogenicity in vivo. Finally, the cysteine-based modification rendered the $UBE2V_{43-51}$ peptide an artificial Ag and strongly stimulated the immune responses against it. Although we currently have no clear explanation for our observations, our findings should contribute to our understanding of what kinds of immune response might be elicited in vaccinated patients.

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Vaccination with Predesignated or Evidence-Based Peptides for Patients with Recurrent Gynecologic Cancers

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Abstract: Two different trials of peptide vaccination were conducted for patients with recurrent gynecologic cancers. In the first regimen, four HLA-A24⁺ patients (two with cervical cancer and two with ovarian cancer) were vaccinated with peptides that were predesignated before vaccination. Three patients exhibited with a grade 1 adverse effect, and no clinical response was observed in any patients. In the second regimen, six HLA-A24⁺ and four HLA-A2⁺ patients (five with cervical cancer, one with endometrial cancer, one with uterine sarcoma, and three with ovarian cancer) were vaccinated with peptides (maximum four) to which preexisting cytotoxic T lymphocyte precursors in the periphery were confirmed before vaccination. With this regimen, grade 1 adverse effects were observed in eight patients, a grade 2 adverse effect in one patient, and a grade 3 adverse effect (ie, rectal bleeding) in one patient. However, this regimen was able to enhance peptide-specific cytotoxic T lymphocytes in seven of ten patients, and three of five cervical cancer patients showed objective tumor regression. Analysis of immunoglobulin G -reactive to administered peptides suggested that the induction of peptide-specific immunoglobulin G was correlated with clinical responses. Overall, these results suggest that peptide vaccination of patients showing evidence of preexisting peptide-specific cytotoxic T lymphocyte precursors could be superior to vaccination with predesignated peptides, and that the evidence-based regimen is applicable for clinical trials in treatment of patients with recurrent gynecologic cancers.

Key Words: peptide, vaccination, immunotherapy, gynecologic cancer

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Recent advances in molecular biology and tumor immunology have resulted in identification of many tumor antigens and epitopes recognized by tumor-reactive cytotoxic T lymphocytes (CTLs).[1,2] In the field of gynecology, vaccination has been conducted with human papilloma virus (HPV)16 E7-derived peptides for HLA-A2⁺ patients with cervical cancer, although clinical responses have been unsatisfactory.[3–5] We previously identified a panel of antigenic peptides having the potential to induce peptide-specific and tumor-reactive CTLs in patients with epithelial cancers,[6–16] and several antigens have been shown to be expressed in gynecologic cancers and to have the potential to induce CTLs reactive to gynecologic cancers.[17]

In most protocols of peptide-based vaccination, no consideration has been paid to whether or not peptide-specific CTL precursors are preexistent in cancer patients. Since priming of naive CTLs generally takes longer than boosting of primed CTLs, vaccination with peptides after confirmation of preexisting peptide-specific CTL precursors might be therapeutically beneficial because it would promptly induce peptide-specific CTLs. To put this idea into practice, we developed a new culture protocol to screen a panel of antigenic peptides with a limited number of peripheral blood mononuclear cells (PBMCs),[18] and we confirmed that peptide-specific CTL precursors can be detected in most patients with pancreatic or gastric cancer.[19,20] In this study, gynecologic cancer patients were vaccinated with peptides according to two different regimens: vaccination with predesignated peptides or vaccination with peptides to which preexisting CTL precursors in the periphery were confirmed before vaccination. Our results suggest that the latter evidence-based regimen is effective for patients with recurrent gynecologic cancers, especially cervical cancer.

MATERIALS AND METHODS

Patients and Eligibility Criteria

Two different regimens were approved by the Institutional Ethical Review Boards of Kurume University. Com-

plete written informed consent was obtained from all patients at the time of enrollment. According to the protocol, patients were required to be positive for either HLA-A2 or HLA-A24. The expression of HLA-A24 or HLA-A2 molecules on PBMCs of cancer patients was first determined by flow cytometry, and HLA-A2 subtypes were determined by the sequence-specific oligonucleotide probe method. All patients were pathologically confirmed to have gynecologic cancer (cervical cancer, endometrial cancer, uterine carcinosarcoma, or ovarian cancer). Eligibility criteria included an age of 85 years or younger, serum creatinine of <1.4 mg/dL, bilirubin of <1.5 mg/dL, platelet count of ≥100,000/μL, hemoglobin of ≥8.0 g/dL, total WBC of ≥3000/μL, and negativity for hepatitis B and hepatitis C antigens. All patients had been untreated for at least 4 weeks before the study, and had an Eastern Cooperative Oncology Group performance status of 0 to 1. Patients with evidence of serious illness, an active secondary malignancy during five years before entry, immunosuppression, or autoimmune disease were excluded from the study.

Screening of Peptide-Specific CTL Precursors

Thirty milliliters of peripheral blood was obtained before and after the third, sixth, ninth, and twelfth vaccinations, and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method.[18] Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of a peptide in 200 μL of culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (GIBCO BRL), 10% FCS, 100 U/mL of interleukin-2 (IL-2), and 0.1 μM MEM nonessential amino acid solution (GIBCO-BRL). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) every three days. After incubation for twelve days, these cells were harvested and tested for their ability to produce IFN-γ in response to CIR-A2402 or T2 cells that were preloaded with either a corresponding peptide or HIV peptides (RYLRQQLLGI for HLA-A24 and LLFGYPVYV for HLA-A2) as a negative control. The level of IFN-γ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/mL). All assays were performed in quadruplicate. A two-tailed Student's *t* test was used for the statistical analyses. Based on the results of this test, up to four positive peptides were selected for each patient, and then a skin test was performed. Peptides, which were negative for the skin test, were vaccinated into cancer patients. To evaluate the effects of immunization and newly determined peptides for vaccination, patients were re-screened for peptide-specific CTL precursors after the sixth and the twelfth vaccinations.

Peptides and Vaccination

The peptides used in the present study were prepared by Multiple Peptide Systems (San Diego, CA) under the conditions of Good Manufacturing Practice. The sequences of the peptides are shown in Table 1. All of these peptides have previously been shown to induce HLA-A24- or HLA-A2-restricted and tumor-reactive CTLs in PBMCs of cancer patients.[6–16] Although all peptides for HLA-A2$^+$ patients were selected based on the binding motif to HLA-A*0201 molecules, these peptides are immunogenic not only in HLA-A*0201 patients but also in those with other HLA-A2 subtypes such as HLA-A*0206 or HLA-A*0207.[13–16] Montanide ISA-51 adjuvant was manufactured by Seppic, Inc. (Franklin Lake, NJ). The peptides were supplied in vials containing three mg/mL sterile solution for injection. Three milligrams of peptide with sterile saline was added in a 1:1 volume to the Monotide ISA-51 and then mixed in a Vortex mixer (Fisher Inc., Alameda, CA). The resulting emulsion was injected subcutaneously in the lateral thigh using a glass syringe. Patients were vaccinated initially with three injections every two weeks to determine the toxicity levels. For the patients with no toxicity, the vaccinations were then given every two weeks after obtaining additional informed written consent.

Delayed-type Hypersensitivity (DTH) Skin Test

A skin test was performed using 50 μg of each peptide injected intradermally in a volume of 100 μL using a tuberculin syringe and a 27-gauge needle. Saline was injected as a negative control. Patients were examined 48 hours after the injection and were considered to be positive if they showed an at least 10-mm-diameter induration or erythema.

^{51}Cr-Release Assay and Targets

Cytotoxic activity was measured using a standard 6-hour ^{51}Cr-release assay.[21] In brief, cryopreserved PBMCs were thawed and cultured in the culture medium. On the 14th day of culture, the cells were harvested and used for the assay. Targets used for the ^{51}Cr-release assay were as follows: SKG-I (HLA-A24$^+$ cervical cancer cells), TOC-2 (HLA-A2$^+$ ovarian cancer cells), QG56 (HLA-A24$^-$ lung cancer cells), and HLA-A2$^+$ PHA-blastoid T cells. To minimize nonspecific killing, 20-fold unlabeled K562 cells were added to each well.

Kinetics of Peptide-Specific CTL Precursors

For kinetic analysis of peptide-specific CTL precursors, pre- and post-vaccination PBMCs were incubated at 1×10^5 cells per well in 96-well U-bottom microculture plates in the presence of a peptide. Cells from each well were harvested at the 14th day of culture and tested for their ability to produce IFN-γ by recognition of peptide-pulsed C1R-A24 or T2 cells. The criteria for positive wells are given in the legend for Table 2.

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TABLE 1. Pre-vaccination Screening of Peptide-Specific CTL-Precursors

Peptide	Sequence	Reference	Patient						Positive	Vaccinated Case
<HLA-A24>			EBG-001	EBG-002	EBG-003	EBG-004	EBG-006	EBG-007		
SART1 690	EYRGFTQDF	6	● Ar	○ B	○ B				3/6	2/6
SART2 93	DYSARWNEI	7		○ C		○ AC			2/6	2/6
SART2 161	AYDFLYNYL	7	○ A			○ C	○ Ar		3/6	3/6
SART2 899	SYTRLFLIL	7	○ B		○ A			● E	3/6	2/6
SART3 109	VYDYNCHVDL	8	● A		○ A		○ C		3/6	2/6
SART3 315	AYIDFEMKI	8							0/6	0/6
CypB 84	KFHRVIKDF	9				● A			1/6	0/6
CypB 91	DFMIQGGDF	9			○ B	○ E	○ E		3/6	3/6
lck 208	HYTNASDGL	10	○ A	○ A				○ D	3/6	3/6
lck 486	TFDYLRSVL	10		○ A		○ CCC		○ D	3/6	3/6
lck 488	DYLRSVLEDF	10		● Ar					1/6	0/6
ART1 170	EYCLKFTKL	11					● ArA		1/6	0/6
ART4 13	AFLRHAAL	12			● B			● B	2/6	0/6
ART4 75	DYPSLSATDI	12	○ B				○ E		2/6	2/6
<HLA-A2>				EBG-101	EBG-102	EBG-103	EBG-194			
SART3 302	LLQAEAPRL	13			○ AA				1/4	1/4
SART3 309	RLAEYQAYI	13				● CC			1/4	0/4
CypB 172	VLEGMEVV	14		● Ar					1/4	0/4
CypB 129	KLKHYGPGWV	14							0/4	0/4
lck 246	KLVERLGAA	15				○ A			1/4	1/4
lck 422	DVWSFGILL	15			○ A		○ A		2/4	2/4
MAP 294	GLLFLHTRT	16		○ AC			○ AC		2/4	2/4
MAP 432	DLLSHAFFA	16		○ ABC		○ C	○ AAC		3/4	3/4
WHS 103	ASLDSDPWV	16		○ ArA					1/4	1/4
WHS 141	ILGELREKV	16				○ AC			1/4	1/4
UBE 43	RLQEWCSVI	16							0/4	0/4
UBE 85	LIADFLSGL	16							0/4	0/4
UBE 208	ILPRKHHRI	16							0/4	0/4
HNR 140	ALVEFEDVL	16							0/4	0/4
HNR 501	NVLHFFNAPL	16		○ CC	○ A	○ A	○ AA		4/4	4/4
EIF 51	RIIYDRKFL	16								

White circles indicate that the peptide was positive for the CTL-precursor induction assay and was vaccinated. Black circles indicate that the peptide was positive for the CTL-precursor induction assay but was not administered due to immediate-type hypersensitivity by skin test.
The assay was performed in quadruplicate and was evaluated by the criteria shown in Table 2.
The classification is shown as alphabet and each character represents the result of each well. For example, ABC means that three wells were judged as A, B, C and one well was negative quadruplicate wells.

Detection of Peptide-Specific Immunoglobulin G (IgG)

The serum levels of peptide-specific IgG were measured by ELISA as previously reported.[22] Briefly, a peptide (20 μL/well)-immobilized plate was blocked with BlockAce (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween 20-PBS. One hundred microliters per well of serum samples diluted with 0.05% Tween 20-BlockAce were added to the plate. After a 2-hour incubation at 37°C, the plate was washed and further incubated for two hours with a 1:1000-dilluted rabbit anti-human IgG (γ-chain-specific: DAKO, Glostrup, Denmark). The plate was washed again, 100 μL of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish (En Vision, DAKO) was added to each well, and the plate was incubated for 40 minutes. The plate was washed once again, 100 μL/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of one M phosphoric acid. To estimate peptide-specific IgG levels, the optical density (OD) values of each sample were compared with those of serially diluted standard samples, and

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TABLE 2. Classification of CTL Response to Peptides

Classification	P Value*	IFN-γ Production†
Ar (armed response)	≤0.1	500 ≤ value
A	≤0.05	50 ≤ value
B	≤0.05	25 ≤ value
C	0.05 < P ≤ 0.01	50 < value
D	0.05 < P ≤ 0.01	25 ≤ value ≤ 50
E	0.1 < P ≤ 0.03	100 ≤ value

*The P value was determined by Student's t test.
†Specific IFN-γ production (pg/mL) was calculated by subtracting the response to HIV-derived irrelevant peptide.

the values were shown as OD units per milliliter. To confirm the specificity of IgG to the peptide, we cultured 100 μL of sample in the peptide-coated wells to absorb peptide-specific IgG, and determined the levels of peptide-specific IgG in the resultant sample.

Evaluation of Response to Treatment

All known sites of disease were evaluated every three months by CT scan or MRI, and/or x-ray examination before and after vaccinations. However, additional examinations were performed when the clinical conditions changed. Patients were assigned a response category according to the response evaluation criteria in solid tumors, based on the June 1999 revision of the WHO criteria published in the WHO Handbook for Reporting Results of Cancer Treatment. Tumor size was evaluated by the longest diameter, and tumor regression of more than 30% for four weeks was regarded as a partial response (PR). The levels of tumor markers, including CA125, CA19-9, carcinoembryonic antigen, and SCC, were measured at the Clinical Examination Laboratory at Kurume University.

RESULTS

Demographics of Patients

Four and 10 patients with gynecologic cancers were enrolled in two different vaccination regimens, respectively. The demographic details of the patients are shown in Table 3. The median age of these patients was 53.5 years (range, 38–68 years). Four HLA-A24$^+$ patients (2 with cervical cancer and 2 with ovarian cancer) were enrolled in the first vaccination regimen, in which predesignated peptides were vaccinated based on the finding that SART2 and ART4 antigens were identified using HLA-A24-restricted CTLs reactive to squamous cell carcinoma and adenocarcinoma, respectively.[7,12] The other 6 HLA-A24$^+$ and 4 HLA-A2$^+$ patients (five with cervical cancer, three with ovarian cancer, one with endometrial cancer,

TABLE 3. Patient Characteristics

Regime	Case No.	HLA	Age (yr)	PS*	Tumor	Stage	Site of Metastasis	Previous Treatment†	No. of Vaccination Received	Clinical Response
1	SART2-001	A24	67	0	Cervical cancer	IVa	Pelvic LN	C/R, C	9	PD (3M)
1	SART2-002	A24	52	1	Cervical cancer	IIb	Lung, para-aorta LN, pelvic LN, virchow LN	S, C, R	9	PD (5M)
1	ART4-001	A24	52	0	Ovarian cancer	IIc	Multiple liver, multiple LN	S, C	7	PD (1M)
1	ART4-002	A24	68	0	Ovarian cancer	IIIa	Lung, ischial bone	S, C, R	15	PD (3M)
2	EBG-001	A24	40	0	Cervical cancer	Ib	Para-aorta LN	C/R	31	SD (3M), PR (4M), PD (15M)
2	EBG-002	A24	67	0	Endometrial cancer	Ic	Lung	S, C	18	PD (2M)
2	EBG-003	A24	66	0	Cervical cancer	IIb	(−)	S, C	25	SD (3M), PD (6M)
2	EBG-004	A24	57	0	Ovarian cancer	IIIc	(−)	S, C	13	SD (8M)
2	EBG-006	A24	56	0	Uterine carcinosarcoma	III	(−)	S	8	SD (3M), PD (5M)
2	EBG-007	A24	38	0	Cervical cancer	IIIb	Lung	C/R, C	7	PD (2M)
2	EBG-101	A2 (A*0206)	67	0	Cervical cancer	IVa	(−)	C, R	10	SD (3M), PR (10M)
2	EBG-102	A2 (A*0206)	49	0	Ovarian cancer	IIIc	(−)	S	8	PD (3M)
2	EBG-103	A2 (A*0201)	63	0	Cervical cancer	IVa	Parametrium	R	14	SD (13M)
2	EBG-104	A2 (A*0201)	59	1	Ovarian cancer	IIIc	Spleen	S, C	5	PD (2M)

*Performance Status by ECOG score.
†S, surgery; C, chemotherapy; R, radiation therapy; C/R, chemoradiotherapy; LN, lymph node.

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and one with uterine sarcoma) were enrolled in the second regimen, in which patients were vaccinated with peptides to which preexisting CTL precursors were confirmed before the peptide vaccination. Three patients (EBG-003, EBG-004, and EBG-006) had no measurable disease at the time of entry but were enrolled in this study because they had high risk of relapse (EBG-003, cervical cancer stage IIb post chemotherapy-radiotherapy; EBG-004, ovarian cancer stage IIIc recurrence postchemotherapy; and EBG-006, uterine carcinosarcoma stage III post simple total abdominal hysterectomy and bilateral salpingo-oophorectomy). No patient had received any concurrent treatments, or any steroids, or any other immunosuppressive drugs, for 4 weeks prior to the vaccination. All 10 patients completed the first three vaccinations within the protocol under informed consent, and all of them received additional vaccinations (5 to 31) after providing additional informed consent.

Toxicities

All patients were evaluated for toxicity levels. The overall toxicities are shown in Table 4. In the first regimen, local redness and swelling were observed in 3 of 4 patients, and no other toxicity was observed. In the second regimen, common adverse events were local redness and swelling (grade 1 or 2) at the injection sites. Fever was observed in 3 patients. Inguinal lymph node swelling (grade 1) was observed in one patient. Although rectal bleeding (grade 3) was observed in one patient (EBG-101) after the fifth vaccination, the correlation to the vaccination was unclear because this patient had radiation-induced colitis in the rectum before entry into this trial. In addition, there was no clinical evidence of autoimmune reactions as determined by symptoms, physical examination, or laboratory tests.

First Regimen

In the first regimen, patients were vaccinated with predesignated peptides. Although we had designated 3 SART2 peptides (SART2 93, SART2 161, and SART2 899) as peptides for two patients with cervical cancer (SART2-001 and SART2-002), the SART2 899 peptide was not vaccinated because this peptide elicited immediate-type hypersensitivity at the skin test (data not shown). The other two patients with ovarian cancer (ART4-001 and ART4-002) were vaccinated with only the ART4 75 peptide because the ART4 13 peptide also elicited immediate-type hypersensitivity at the skin test (data not shown).

Screening of Peptide-Specific CTL Precursors and the Second Regimen

In the second regimen, prevaccination PBMCs were used for screening of preexisting CTL precursors reactive to peptides. Fourteen peptides binding to HLA-A24 molecules and 16 peptides binding to HLA-A2 molecules were used for the screening. The results for each well were classified into 6 groups based on the P value and the amounts of IFN-γ, as shown in Table 2. Up to 4 peptides were selected as candidates for the peptide vaccination. Patients who showed immediate-type hypersensitivity by the skin test were vaccinated with the fifth-ranked peptide, provided that their skin test result for this peptide was negative. The results of prevaccination screening of peptide-specific CTL precursors are shown in Table 1. In HLA-A24$^+$ patients, the SART2 161, the CypB 91, the lck 208, and the lck 486 peptides were most frequently positive (3 of 6 patients) for CTL precursors without immediate-type hypersensitivity. In HLA-A2$^+$ patients, the HNR 501 peptide was positive in all patients, and the MAP 432 peptide showed the second highest rate of positivity (3 of 4 patients). It is of note that 8 patients were positive for at least 4 peptides, with the exception that EBG-007 and EBG-102 were positive for 2 and 3 peptides, respectively. After the 6th vaccination, peptide-specific CTL precursors were rescreened, and peptide candidates for additional vaccination were determined. In some cases, peptide-specific CTL precursors were screened a third time after the twelfth vaccination, and further peptide candidates for vaccination were determined. All data are summarized in Table 5. The peptide vaccination based on the second regimen augmented peptide-specific IFN-γ production in 7 patients. Unexpectedly, peptide vaccination seemed to induce CTLs reactive to irrelevant peptides. As typically observed in patient EBG-103, the first vaccination with the lck 246, the

TABLE 4. Toxicity Associated With the Peptide Vaccination

Toxicity	Regimen 1					Regimen 2				
	Grade 1	Grade 2	Grade 3	Grade 4	Total	Grade 1	Grade 2	Grade 3	Grade 4	Total
Dermatologic	3				3/4	4	1			5/10
Fever					0/4	3				3/10
Rectal bleeding					0/4			1		1/10
Inguinal lymph node swelling					0/4	1				1/10

Toxicities are based on NIH Common Toxicity Criteria.

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TABLE 5. Summary of Responses of the Regimen 2

Case	Peptide	Peptide-specific CTL*			Ab to Peptides		DTH		Clinical Response/Time to Progression (Months)
		Pre	Post (6th)	Post (12th)	Pre	Post†	Pre	Post	
<HLA-A24>									
EBG-001	SART2 161	A‡	–	–	–	–	–	–	shrinkage of metastatic LN (42%)
	SART2 899	B	–	A	+	+ (6)	–	–	CEA; 207 → 105
	lck 208	A	Ar	AC	–	+ (9)	–	–	SD (3M), PR (4M), PD (15M)
	ART4 75	B	C	CCC	–	+ (21)	–	–	
	SART1 690	Ar	C	ArAr	–	+ (12)	–	–	
	SART3 109	AA	Ar	–	–	+ (5)	–	–	
	lck 488	–	A	–	–	+ (5)	–	–	
	CypB 91	–	–	A	–	–	–	–	
EBG-002	SART1 690	B	–	–	–	–	–	–	PD/2M
	SART2 93	C	–	AA	–	–	–	–	
	lck 208	A	B	–	–	+ (9)	–	–	
	lck 486	A	–	A	–	+ (6)			
	ART1 170	–	–	–	–	–	–	–	
	lck 488	Ar	–	AAAE	–	–	–	–	
EBG-003	SART1 690	B	A	A	–	–	–	–	SD (3M), PD (6M)
	SART2 899	A	–	–	–	–	–	+ (3)	
	SART3 109	A	–	–	–	–	–	–	
	CypB 91	B	–	ArA	–	–	–	–	
	lck 488	–	AAA	–	–	–	–	+ (9)	
	SART2 93	–	BB	–	–	–	–	–	
	SART2 161	–	A	–	–	–	–	–	
	SART3 315	–	–	Ar	–	+ (10)	–	+ (14)	
EBG-004	SART2 93	AC	AC	A	–	–	–	–	SD (8M)
	SART2 161	C	AAA	AB	–	–	–	+ (3)	
	CypB 91	E	–	–	–	–	–	+ (3)	
	lck 486	CCC	–	AA	–	+ (6)	–	–	
	SART3 315	–	A	AAA	–	–	–	+ (7)	
EBG-006	SART2 161	Ar	ArAr	NT	–	–	–	–	SD (3M), PD (5M)
	SART3 109	C	ArArA	NT	–	+ (6)	–	–	
	CypB 91	E	–	NT	–	–	–	–	
	ART4 75	E	–	NT	–	–	–	–	
	SART2 93	–	ArAr	NT	–	–	–	–	
	lck 488	–	C	NT	–	–	–	–	
EBG-007	lck 208	D	–	NT	–	+ (6)	–	–	PD (2M)
	lck 486	D	–	NT	–	–	–	–	
	SART3 109	–	B	NT	–	+ (11)	–	–	
<HLA-A2>									
EBG-101	MAP 294	AC	–	NT	–	–	–	–	Shrinkage of tumor (48%)
	MAP 432	ABC	ArA	NT	–	+ (6)	–	–	SD (3M), PR (10M)
	WHS 103	ArA	A	NT	–	–	–	–	SCC 289 → 36
	HNR 501	CC	–	NT	–	–	–	–	CEA: 13.3 → 6.6
	lck 246	–	ArAr	NT	–	+ (4)	–	–	
	lck 422	–	ArArAr	NT	–	–	–	–	
	UBE 43	–	–	NT	–	+ (4)	–	–	
EBG-102	SART3 302	AA	A	NT	–	–	–	+ (2)	PD (3M)
	lck 422	A	NT	NT	–	–	–	+ (3)	CA 125: 24000 → 17000
	HNR 501	A	–	NT	–	–	–	–	CA 19-9: 113 → 29.3
	SART3 309	–	AA	NT	–	–	–	–	
	MAP 294	–	A	NT	–	–	–	–	
	MAP 432	–	B	NT	–	–	–	–	
EBG-103	lck 246	A	ArC	ArArA	–	+ (5)	–	–	SD (13M)
	MAP 432	C	–	AACC	–	+ (7)	–	–	
	WHS 141	AC	–	–	–	+ (4)	–	–	
	HNR 501	A	B	A	–	–	–	–	
	lck 422	–	ABCC	AAAA	–	–	–	–	
	UBE 43	–	AAAC	ABC	–	+ (2)	–	–	
EBG-104	lck 422	A	NT	NT	–	–	–	–	PD (2M)
	MAP 294	AC	NT	NT	–	+ (3)	–	–	
	WHS 432	AAc	NT	NT	–	–	–	–	
	HNR 501	AA	NT	NT	–	–	–	–	

NT: not tested.
*The criteria are shown in Table 2.
†The number in the parenthesis represents the vaccination when IgG to the peptide was detected for the first time.
‡Peptides shown as a bold letter were administered into patients.

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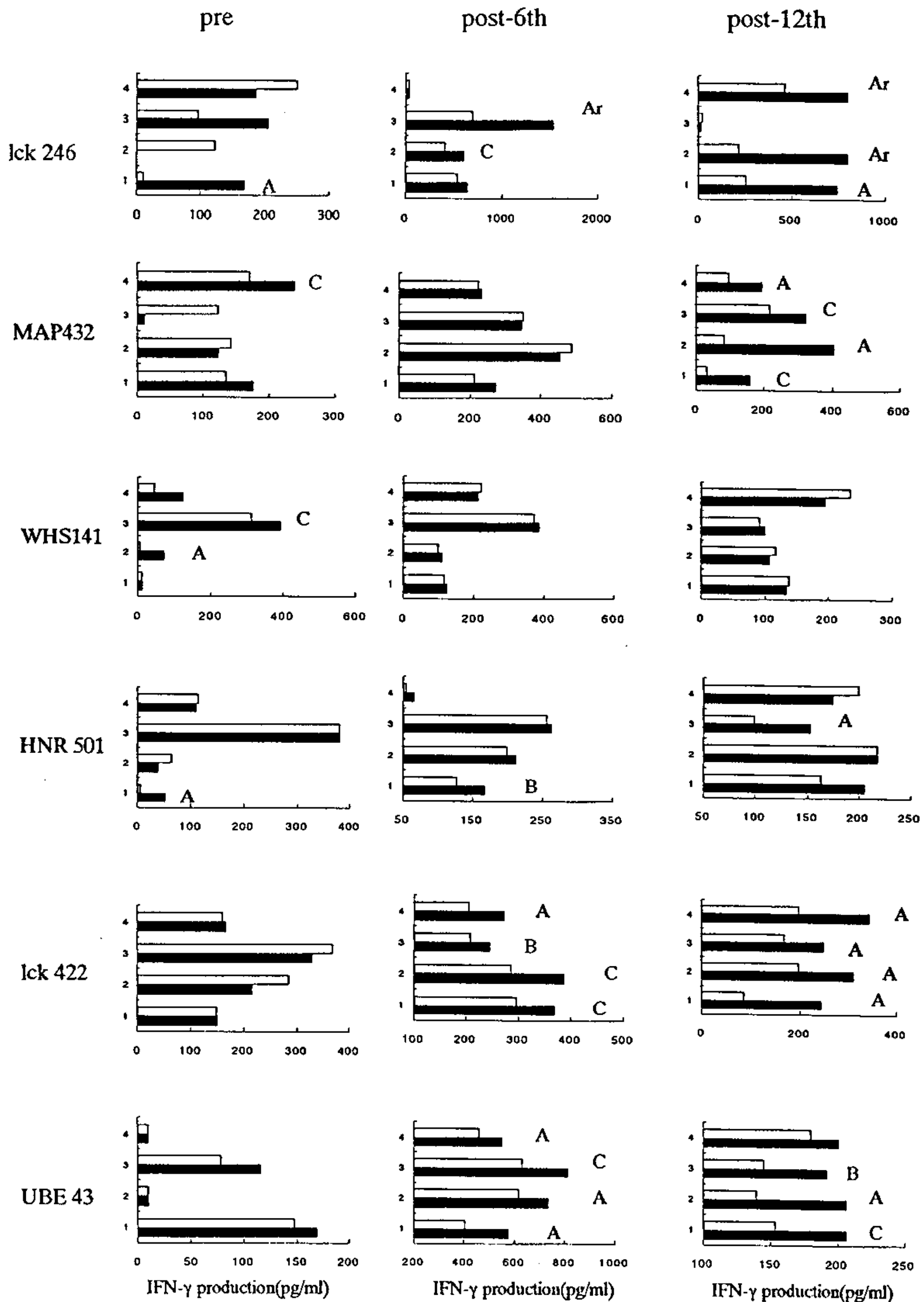

FIGURE 1. Detection of peptide-specific CTL precursors. Pre- and post- (6th and 12th) vaccination PBMCs from patient EBG-103 (HLA-A2+) were applied for the screening of peptide-specific CTL precursors. Values represent IFN-γ production by the in vitro cultured PBMCs. Criteria for evaluation are shown in Table 2. Open and closed bars represent IFN-γ production in response to HIV peptide-pulsed and the corresponding peptide-pulsed stimulator cells, respectively. T2 cells were used as stimulator cells.

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MAP 432, the WHS 141, and the HNR 501 peptides enhanced CTLs reactive to the lck 246 peptide but also resulted in increased responses of CTLs reactive to both the lck 422 and the UBE 43 peptide, neither of which was administered into the patient (Fig. 1). After the third screening, this patient was vaccinated with the lck 246, the HNR 501, the lck 422, and the UBE 43 peptides, but this vaccination augmented CTLs reactive to the MAP 432 peptide. The same effect was found in the other 7 patients. However, no augmentation of peptide-specific CTLs was observed in patient EBG-007. As for patient EBG-104, the assay of CTL precursors before the sixth vaccination was not performed because the patient died after the fifth vaccination due to progression of disease.

Kinetic Assay of Cytotoxicity of the Second Regimen

Because of the limited availability of samples, we kinetically evaluated anti-tumor cytolytic activity of pre- and post-vaccination PBMCs in only four patients (Fig. 2). In patient EBG-001, CTL activity against SKG-I (HLA-A24$^+$) was augmented transiently after the vaccination, compared with that against QG56 (HLA-A24$^-$) but was subsequently diminished. In patient EBG-007, CTL activity against SKG-I increased after the sixth vaccination. In patient EBG-101, CTL activity against TOC-2 (HLA-A2$^+$) was transiently augmented compared with that against HLA-A2$^+$ PHA blasts. In patient EBG-102, CTL activity against TOC-2 was higher than that against HLA-A2- QG-56 throughout the peptide vaccination.

Serum IgG Specific to Peptides Administered

We also examined whether peptide-specific IgG could be detected in vaccinated patients (Table 5). Peptide-specific IgG was detected before vaccination in only patient, EBG-001. Although no peptide-specific IgG was detected in the other 9 patients before the peptide vaccination, peptide vaccination resulted in the induction of peptide-specific IgG in 9 of 10 pa-

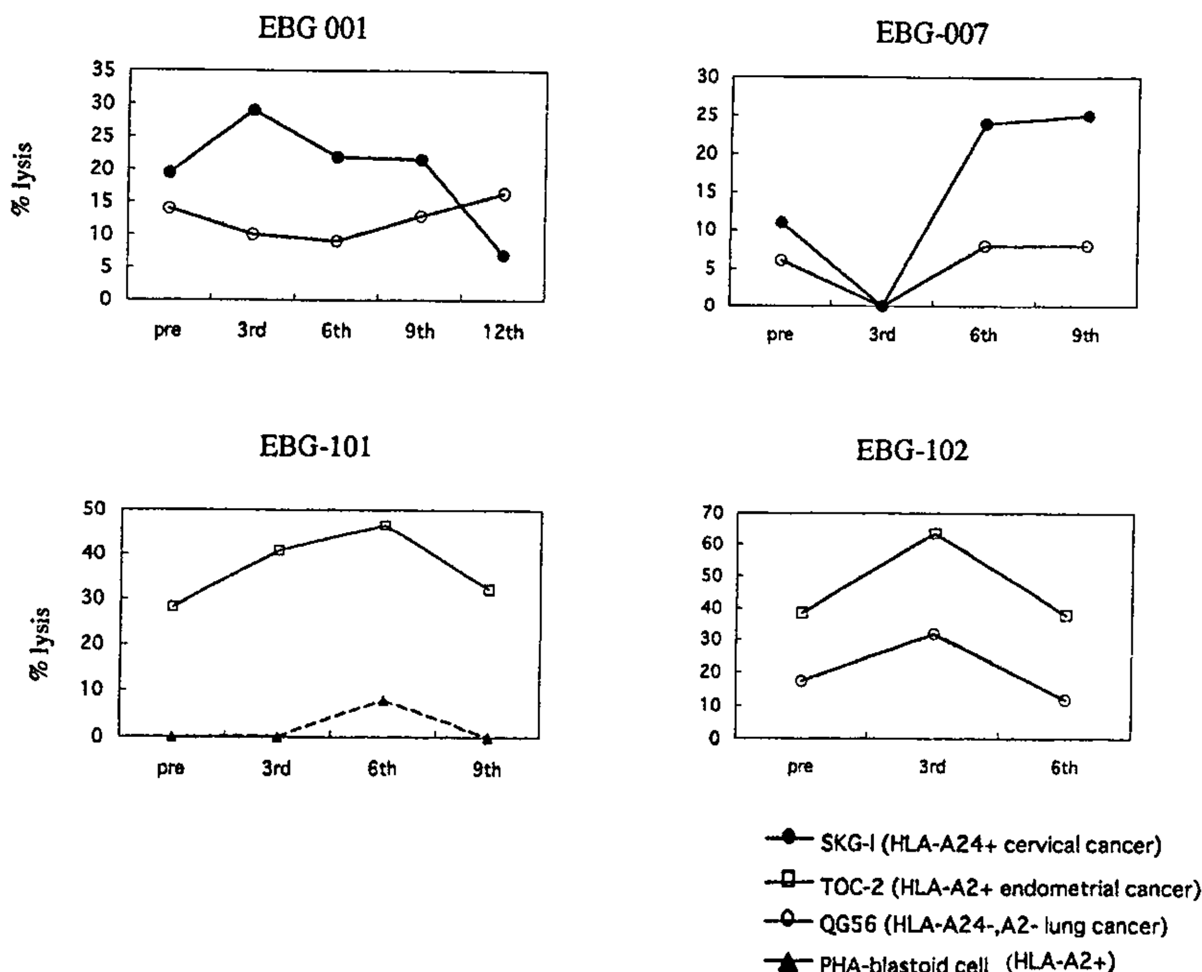

FIGURE 2. Kinetic analysis of cytotoxicity of vaccinated patients. Frozen pre- and post-vaccination (3rd, 6th, 9th, and 12th) PBMCs were thawed and incubated for 14 days with IL-2 alone without any peptides. A cytolytic assay against targets was carried out by a 6-hour ^{51}Cr-release assay at an E/T ratio of 40:1. Values are the means of triplicate assays. Patients EBG-001 and EBG-007 were positive for HLA-A24, and patients EBG-101 and EBG-102 were positive for HLA-A2.

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tients. Representative results for patients EBG-001, EBG-002, EBG-004, EBG-006, and EBG-101 are shown in Figure 3a. In patients EBG-001 and EBG-002, peptide-specific IgG was detected after the ninth vaccination. In patients EBG-004, EBG-006, and EBG-101, 6 vaccinations were sufficient to elicit peptide-specific IgG. As shown in Figure 3b, peptide-specific IgG in the plasma of patient EBG-001 was absorbed by culturing on peptide-coated plates in an antigen-specific manner. Namely, the reactivity to lck 208 or SART3 109 peptide was diminished by culturing on plates coated with the relevant peptides, but not on those coated with irrelevant peptides.

DTH Skin Test

No DTH reaction against peptides was observed before vaccination in any patients (Table 5). Peptide-specific DTH reactions were observed in three patients after the vaccination in the second regimen. In the first patient, EBG-003, DTH reaction to each of the SART2 899, the SART2 93, and the SART3 315 peptides was observed after the peptide vaccination. Another patient, EBG-004, exhibited the DTH reaction against the SART2 161, CypB 91, and SART3 315 peptides, while patient EBG-102 exhibited the DTH reaction against the SART3 302 and lck 422 peptides after the second and third vaccinations, respectively.

Clinical Responses

As shown in Table 3, the first regimen failed to induce any clinical response in the four patients, all of whom showed progressive disease. In the second regimen, among the seven patients who had measurable disease at entry, two patients (EBG-001 and EBG-101) and two patients (EBG-004 and EBG-103) showed PR and stable disease, respectively (Tables 3 and 5). As shown in Figure 4a, tumor regression was observed in patient EBG-001, who had para-aortic lymph node metastasis, and that lesion was reduced 42% between the tenth and 17th vaccinations, with the level of carcinoembryonic an-

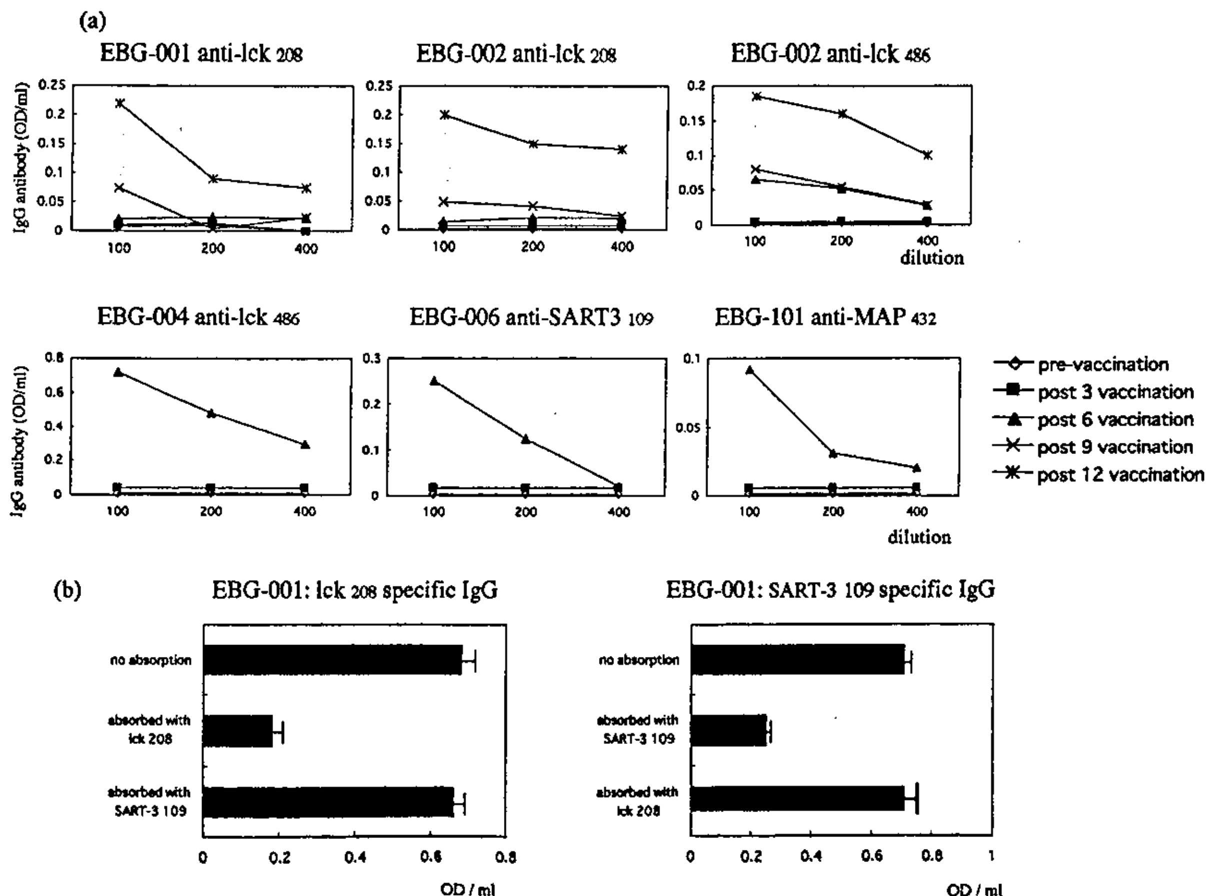

FIGURE 3. Serum IgG reactive to vaccinated peptides. a: The levels of peptide-specific IgG of pre- and post-vaccination plasma from 5 patients were determined by ELISA. b: Plasma from patient EBG-001 after the 12th peptide vaccination was cultured with the indicated peptide-coated plates. Thereafter, the levels of IgG reactive to relevant peptides in the resultant samples were determined by ELISA.

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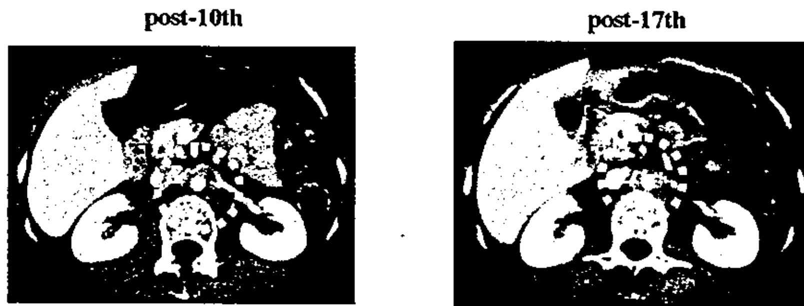
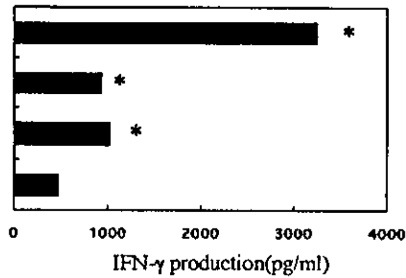
(a) [EBG-001]

post-10th post-17th



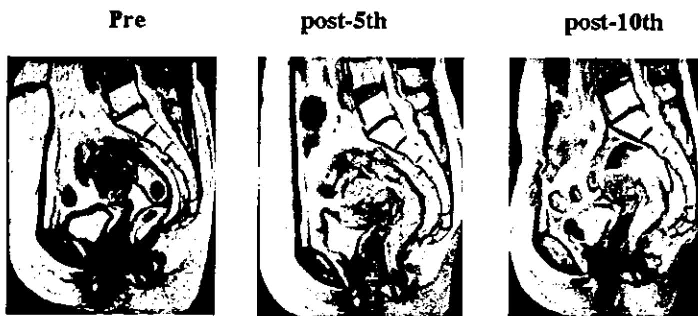
	post-10th	post-17th
Shrinkage of tumor		42%
Level of CEA(ng/ml)	188	105

SKG-I (HLA-A24+)
HCS (HLA-A24+)
TCS (HLA-A24+)
OMC-I (HLA-A24-)



(b) [EBG-101]

Pre post-5th post-10th



	Pre	post-5th	post-10th
Shrinkage of tumor		26%	48%
Level of SCC (ng/ml)	273	289	36

(c) [EBG-103]

pre post-21st

	pre	post-21st
Shrinkage of tumor		13%

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tigen decreasing from 207 to 105. This patient received irradiation to the para-aortic lymph node metastases two months before the peptide vaccination but did not receive irradiation to the primary lesion. This patient was judged as PR four months after the peptide vaccination. In addition, in vitro cultured inguinal LN cells, which were draining from the vaccination site, produced a higher level of IFN-γ in response to HLA-A24$^+$ tumor cells than in response to HLA-A24$^-$ tumor cells. Patient EBG-101, with recurrent cervical cancer invading to uterine body, also showed objective tumor shrinkage. The MRI results of patient EBG-101 at prevaccination and after the fifth and 10th vaccinations are shown in Figure 4b. This patient was diagnosed as showing a PR for 10 months. In this patient, the levels of squamous cell carcinoma (SCC)-related antigen and carcinoembryonic antigen decreased from 289 and 13.3 to 36 and 6.6, respectively. This patient received irradiation to the lesion 5 years before the peptide vaccination. Patient EBG-103 was not judged as PR because the evaluated longest tumor diameter showed a 13% reduction, although the tumor invading to the parametrium drastically shrunk after the peptide vaccination (Fig. 4c). This patient received irradiation to the lesion 2 months before the peptide vaccination. Patient EBG-102 showed progressive disease 3 months after the vaccination, but the levels of tumor markers were significantly decreased: the levels of CA125 and CA19-9 decreased from 24,000 and 113 to 17,000 and 29.3, respectively. Three patients (EBG-002, EBG-007, and EBG-104) were diagnosed with progressive disease at two months after the vaccination. Although the remaining three patients (EBG-003, EBG-004, and EBG-006) had no measurable disease at entry, they were enrolled in this study because of their high risk of recurrence, and they agreed to enter to the trial for purpose of prophylaxis. Patient EBG-004 showed stable disease for eight months, but patients EBG-003 and EBG-006 were diagnosed with progressive disease. In most cases, patients received the peptide vaccination of the second regimen as outpatients, and the performance status remained good throughout the treatment period.

DISCUSSION

We previously identified a panel of antigenic peptides capable of inducing tumor-reactive CTLs in HLA-A24$^+$ or HLA-A2$^+$ patients.[6–16] In a subsequent study, we vaccinated some of these peptides into cancer patients, but induction of cellular responses to either peptides or cancer cells was insufficient in the postvaccination PBMCs.[23] In this study, we conducted two different regimens for patients with gynecologic cancers. In the first regimen, 4 HLA-A24$^+$ patients were vaccinated with either SART2-derived or ART4-derived peptides, which were predesignated before the vaccination. Although the vaccination protocol was completed safely, no objective response was observed. However, in the second regimen, 6 HLA-A24$^+$ and 4 HLA-A2$^+$ patients were vaccinated with peptides to which CTL precursors were preexisting before vaccination. Increased cellular responses to the vaccinated or nonvaccinated peptides were observed in the postvaccination PBMCs of 7 of 10 patients tested. In addition, increased humoral responses to the vaccinated peptides were observed in the postvaccination plasma of nine of ten patients tested. Three patients with cervical cancer showed objective tumor regression. These lines of evidence indicate that the second, evidence-based peptide vaccination is feasible and superior to the first, predesignated peptide vaccination for treatment of patients with recurrent gynecologic cancers, especially cervical cancer.

In the evidence-based vaccination, the efficacy of the peptide vaccination was evaluated by several methods. We evaluated the reactivity of CTLs using ELISA for IFN-γ and found that peptide-specific IFN-γ-producing CTLs could be induced by the peptide vaccination with higher incidence. We applied a cytolytic assay and found that the evidence-based peptide vaccination could enhance the cytotoxicity of CTLs, although only a small number of patients were examined in this study. We also measured the levels of IgG reactive to the administered peptides. The results showed that peptide-specific IgG was elicited in most cases. We checked the DTH reaction to administered peptides but found that DTH reactions were induced in only 3 of 10 patients. The results from these different methods did not appear to be correlated. However, two assays (ie, the assay of peptide-specific IFN-γ production and that of peptide-specific IgG induction) appeared to be useful in evaluating the efficacy of peptide vaccination, since these two responses, but not the DTH response, were observed in 3 cases (EBG-001, EBG-101, and EBG-103) that showed objective tumor regression or long stable disease. Cytolytic activity against cancer cells was also enhanced in patients EBG-001 and EBG-101. Although DTH response is generally considered useful in monitoring peptide-specific immune response, this was not the case in the present trial. Although recent reports indicate that ELISPOT assay is a useful method to monitor peptide-specific T cell responses,[24,25] we did not carry out

FIGURE 4. Tumor regression in 3 patients. **a:** The size of the para-aortic LN with metastasis of patient EBG-001 was evaluated using CT scan. The level of carcinoembryonic antigen in serum was measured after the 10th and 17th vaccination. Inguinal LN cells with metastasis of patient EBG-001 were cultured with IL-2 (100 U/mL) for 14 days, and IFN-γ production in response to 4 kinds of gynecologic cancer cell lines was determined by ELISA. $^*P < 0.05$ statistically significant compared with a control. **b:** Patient EBG-101 was kinetically evaluated for the size of tumor mass using MRI. The levels of SCC in the serum were kinetically measured. **c:** Patient EBG-103 was kinetically evaluated for the size of tumor mass using MRI.

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this assay in this study. Further studies will be needed to determine which methods are the most useful in monitoring peptide-specific T cell responses.

In the present study, we assessed peptide-specific CTL responses based on a classification consisting of two parameters: the *P* value and IFN-γ release. The main reason for using this classification was that the level of IFN-γ produced by peptide-specific CTLs varied among quadruplicate wells. It is possible that one well may have contained peptide-specific CTL precursors, whereas another may have contained none. Another reason was that we had to examine the presence of CTL precursors specific to 14 or 16 different kinds of peptides using the limited number of PBMCs from cancer patients. We considered that each well should be individually estimated to screen for the presence of peptide-specific CTL precursors.

All of the peptides used in this study were derived from nonmutated self-antigens involved in cellular proliferation, whereas the most common adverse events of this clinical study were inflammatory reactions at the vaccination site. Fever was also frequently observed. One patient (EBG-101) developed rectal bleeding after the 6th vaccination, but obvious correlation to the peptide vaccination was unclear because this patient had radiation colitis in the rectum before entry into this trial. However, because this patient showed augmented cellular responses after the peptide vaccination, the possibility that the rectal bleeding might have been triggered by an enhanced immune response cannot be excluded.

Because three cervical patients who showed objective clinical responses had received irradiation therapy, there remains the possibility that their responses were caused by irradiation. However, the time intervals between irradiation and the peptide vaccination of patients EBG-101, EBG-102, and EBG-103, were 2 months, 5 years, and 2 months, respectively. In addition, the peptide vaccination was started at least 2 months after radiation therapy, whereas clinical responses had been observed several months after the peptide vaccination and had been continued for more than 10 months. Based on these lines of evidence, we consider that the clinical responses in these three cervical cancer patients were not due to radiation therapy but to the peptide vaccination.

In the evidence-based regimen, peptide-specific IgG was induced in most cases after the peptide vaccination, and clinical responses seemed to be associated with the induction of IgG to the administered peptides. At the present time, we have no idea about what roles IgG plays in the anti-tumor response in cancer patients, or about the results of vaccination-associated IgG induction specific to administered peptides. Peptide-specific IgG might show a direct or indirect anti-tumor effect in cooperation with cellular immunity. $CD4^+$ T cells might participate in the induction of peptide-specific IgG, since in vivo generation of antigen-specific IgG generally requires a cytokine from helper T cells.[26] Information regarding the roles of peptide-specific IgG in peptide-vaccinated patients may contribute to the design of more effective anti-tumor immunotherapy.

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Humoral Responses to Peptides Correlate with Overall Survival in Advanced Cancer Patients Vaccinated with Peptides Based on Pre-existing, Peptide-Specific Cellular Responses

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ABSTRACT

Purpose: **The aim of this study is to find a laboratory marker for overall survival in advanced cancer patients who were vaccinated with peptides based on pre-existing, peptide-specific CTL precursors in the circulation.**

Experimental Design: **A group of 113 patients with advanced cancer (28 colorectal, 22 prostate, 15 lung, 14 gastric, and 34 other cancers) was enrolled in a Phase I clinical study of peptide vaccination in which peptide-specific CTL precursors of prevaccination peripheral blood mononuclear cells were measured, followed by vaccination with these peptides (maximum of four). For cellular responses, pre and postvaccination (sixth) peripheral blood mononuclear cells were provided for measurement of both peptide-specific CTL precursors by IFN-γ release assay and tumor reactivity by ^{51}Cr release assay. Delayed type hypersensitivity was also measured. For humoral response, pre and postvaccination (sixth) sera were provided for measurement of peptide-reactive IgG by an ELISA.**

Results: **The median survival time and 1-year survival rate of the total cases were 346 ± 64.9 days and 44.6%, respectively, and those of patients vaccinated more than six times (n = 91) were 409 ± 15 days and 54.4%, respectively. In these 91 patients, the overall survival of patients whose sera showed increased levels of peptide-reactive IgG (n = 60) was significantly more prolonged (P = 0.0003) than that of patients whose sera did not (n = 31), whereas none of cellular responses correlated with overall survival.**

Conclusions: **Peptide-specific IgG in postvaccination sera could be a suitable laboratory maker for the prediction of prolonged survival in advanced cancer patients vaccinated with peptides based on pre-existing CTL precursors.**

INTRODUCTION

Recent advances in tumor immunology have allowed the identification of a number of antigens and epitopic peptides capable of inducing tumor-reactive CTLs (1–14). Some of these peptides were used for clinical trials, but these initial trials obtained rare clinical responses, as well as dim levels of immune responses to peptides (15–20). One reason for this failure could be an insufficient induction of antitumor responses in these regimens, in which peptide-specific memory T cells were not measured in prevaccination peripheral blood mononuclear cells (PBMCs). Subsequently, we conducted Phase I clinical trials of peptide vaccination in which cancer patients received peptides (a maximum of four) based on information regarding pre-existing, peptide-specific CTL precursors in the circulation (21–25). The other reason for failure might in part be attributable to a lack of an appropriate laboratory marker either to measure immune responses or to predict clinical responses. Regardless of the extensive studies, there are few reproducible and appropriate laboratory markers for prediction of clinical benefits in recently developing peptide-based therapies (15–25) or in the other types of immunotherapies (26–28). In this study, we investigated the correlation of clinical benefits and immune responses to peptides in HLA-A24-positive or -A2-positive cancer patients who were vaccinated with these CTL-directed peptides and reported that humoral responses to peptides correlated with overall survival.

MATERIALS AND METHODS

Trial Eligibility. The ethical review boards of the Kurume University School of Medicine and the Hokkaido University School of Medicine approved the study protocol. Complete written informed consent was obtained from all patients at the time of enrollment. According to the protocol, patients were required to be HLA-A24 positive and/or HLA-A2 positive, have a histologically confirmed lesion of a malignant tumor, have been untreated for ≥4 weeks before the study, and have an Eastern Cooperative Oncology Group performance status of 0–2. Eligibility criteria included an age from 20 to 85 years, a

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creatinine level <1.4 mg/dl, a bilirubin level <1.5 mg/dl, a platelet count of >100,000/mm^3, hemoglobin of >8 grams/dl, and total WBC count of >3000/μm^3. Hepatitis B and C antigens were required to be negative. No patient had received any concurrent treatments, steroids, or any other immunosuppressive drugs for 4 weeks before the initial vaccination. This clinical study was carried out from November 2000 through November 2002.

Peptides and Selection for Vaccination. The peptides used in the present study were prepared under conditions of Good Manufacturing Practice by the Multiple Peptide System (San Diego, CA). The peptide sequences are shown in Table 2. These peptides have the ability to induce HLA-A24- or -A2-restricted and tumor-specific CTL activity in the PBMCs of cancer patients and were frequently expressed on various tumor cell lines (5–14). These peptides were dissolved and stored at −80°C. Stock solutions were diluted with saline just before use. For the peptide screening, prevaccination PBMCs were provided for assays of peptide-specific CTL precursors using methods reported previously (29). Peptide-specific IFN-γ production was calculated by subtraction of IFN-γ production of the peptide-stimulated PBMCs in response to a negative control (HIV peptide) from that in response to a corresponding peptide in quadricate assays, and a two-tailed Student's *t* test was used for the statistical analyses. As reported previously (21–25), positive wells were evaluated in the following order: (*a*) criteria Ar, the peptide-specific IFN-γ production was ≥500 pg/ml, and P was <0.1; (*b*) criteria A, the production was ≥50 pg/ml, and P was <0.05; (*c*) criteria B, 25 ≤ the production <50 pg/ml, and P was <0.05; (*d*) criteria C, the production was ≥50 pg/ml and $0.05 \leq P < 0.1$; and (*e*) criteria D, the production was >100 pg/dl and $0.1 \leq P < 0.2$. According to the results, up to four positive peptides were selected for each patient and were vaccinated as the CTL precursor-oriented peptide vaccine, if an immediate type hypersensitivity reaction against each peptide was not seen in a skin test performed before vaccination. The screening of peptide-specific CTL precursors was also performed by the same method after the sixth vaccination to evaluate the *in vivo* cellular responses to the peptides. Cellular responses to tumor cells in a HLA-A24- or -A2-restricted manner in pre and postvaccination (sixth) PBMCs were measured using a standard ^{51}Cr-release assay whose methods were described elsewhere (19, 20).

Clinical Protocol. Skin tests were performed by intradermal injection of 10 μg of each peptide using a tuberculin syringe and a 26-gauge needle. Saline was used as a negative control for assessment of hypersensitivity. Immediate and delayed type hypersensitivity (DTH) reactions were determined at 20 min and 24 h after the skin test, respectively. At least 5 mm of induration or 10 mm of erythema read 24 h after injection were needed to score the skin test as positive. If immediate type hypersensitivity was negative, the peptide was vaccinated into the patients' s.c. tissue in the site near each tumor's regional lymph nodes, *e.g.*, the upper arm in cases with lung cancer, lateral abdominal wall in cases with gastric cancer, or anterior thigh with the other cancers. Two milliliters of the peptide, which was supplied in vials containing 2 mg/ml sterile solution, were mixed with an equal volume of incomplete Freund's adjuvant (Montanide ISA-51; Seppic, Paris, France) and emulsified in 5-ml sterilized syringes. Three milliliters of each prepared peptide emulsion (maximum of four peptides at one vaccination) were injected s.c. in individual site three times every 2 weeks. For patients showing a favorable clinical course, the vaccinations were continued every 2–4 weeks with informed consent from each patient.

Detection of Serum IgG Levels. An ELISA was used to detect the serum IgG levels specific to the administered peptides, as reported previously (20–25). Briefly, 100 μl/well serum samples diluted with 0.05% Tween 20-Block Ace were added to the peptide (20 μg/well)-immobilized plate, after which the plate was blocked with Block Ace (Yukijirushi, Tokyo, Japan) and washed. After a 2-h incubation, the plate was washed and further incubated for 2 h with a 1:1000-diluted rabbit antihuman IgG (DAKO, Glostrup, Denmark). The plate was washed, and 100 μl of 1:100-diluted goat antirabbit immunoglobulin-conjugated horseradish peroxidase-dextran polymer (EnVision; DAKO) were then added to each well; the plate was incubated for 40 min. After washing, 100 μl/well tetramethyl-benzidine substrate solution (KPL, Guildford, United Kingdom) were added, and the reaction was stopped by the addition of 1 M phosphoric acid. To estimate peptide-specific IgG levels, the absorbance values of each sample were compared with those of serially diluted standard samples, and the values were shown as absorbance. The cutoff value of optimal density (OD) was determined as 0.02 at a serum dilution of 1:100, because the mean of the OD in response to an HIV peptide taken as a negative control was <0.02, as reported previously (21–25). Positive responses were judged by the two criteria. The first criterion is the case if the prevaccination serum showed no reactions, and the postvaccination serum showed at least significant levels of IgG (>0.02 of net OD value at a serum dilution of 1:100) specific to the vaccinated peptides. The other criterion is the case if an OD value of the postvaccination serum showed ≥2-fold increase at a serum dilution of 1:100 than that of the prevaccination serum.

Evaluation of Clinical Responses and Statistical Analysis. All known sites of disease were evaluated by computed tomography scan or X-ray examination. Patients were assigned a response category according to the Response Evaluation Criteria In Solid Tumors (RECIST criteria), the revised version of the WHO criteria published in the WHO Handbook for reporting results of cancer treatment (Geneva, 1979), June 1999 (Final). For prostate cancer patients without measurable lesions, serum prostate-specific antigen levels were used as a marker for evaluation, as reported previously (23, 25). Overall survival was evaluated from the entry date of these clinical trials, regardless of peptide vaccinations after Phase I trials, and analyzed to investigate correlation between clinical benefits and immune responses. Kaplan-Meier curves were described, and survivals were compared using the Log-rank test.

RESULTS

Patients' Characteristics. A group of 113 patients with advanced malignant tumors was enrolled in this vaccination regimen. The types of cancer included the following: (*a*) colorectal cancer (HLA-A24: n = 22, HLA-A2: n = 6); (*b*) prostate cancer (n = 12, 10); (*c*) lung cancer (n = 10, 5); (*d*) gastric

Table 1 Patients characteristics and median survival times

Sort of cancer	n	Age (range)	Male/Female	HLA-A24/-A02	PS[a] 0/1/2	MST ± SE (days)
Colorectal cancer	28	57.5 (27–78)	20/8	22/6	13/12/3	273 ± 70.9
Prostate cancer	22	68.1 (50–85)	22/0	12/10	19/3/0	601 ± 84.1
Lung cancer	15	65.7 (58–74)	9/6	10/5	9/2/4	668 ± 201.2
Gastric cancer	14	64.7 (49–78)	12/2	12/2	3/8/3	139 ± 48.4
Melanoma	7	60.7 (30–76)	4/3	5/2	5/2/0	353 ± 82.5
Cervical cancer	6	50.8 (32–67)	0/6	4/2	6/0/0	324 ± 157.3
Ovarian cancer	3	55.0 (49–59)	0/3	1/2	2/1/0	522 ± 344.6
Breast cancer	3	46.3 (38–58)	0/3	1/2	1/2/0	
Esophageal cancer	3	63.7 (59–70)	2/1	1/2	1/0/2	85 ± 33.1
Uterine cancer	2	61.5 (56–67)	0/2	2/0	2/0/0	415 ± 293.4
Pancreatic cancer	2	61.5 (60–63)	1/1	2/0	1/1/0	165 ± 95.8
Leiomyosarcoma	2	57.5 (54–61)	0/2	2/0	1/0/1	61 ± 43.1
Thyroid cancer	1	62	0/1	1/0	1/0/0	
Chronic lymphocytic leukemia	1	77	1/0	1/0	0/1/0	
Bladder cancer	1	53	1/0	1/0	1/0/0	199
Renal cell carcinoma	1	39	1/0	1/0	1/0/0	
Periurethral cancer	1	55	0/1	0/1	1/0/0	
Seminoma	1	23	1/0	0/1	1/0/0	409
Total	113	61.0 (23–85)	74/39	78/35	68/32/13	346 ± 64.9

[a] PS, performance status; MST, median survival time.

cancer (n = 12, 2); (*e*) melanoma (n = 5, 2); (*f*) cervical cancer (n = 4, 2); (*g*) ovarian cancer (n = 1, 2); (*h*) breast cancer (n = 1, 2); (*i*) esophageal cancer (n = 1, 2); (*j*) uterine cancer (n = 2, 0); (*k*) pancreatic cancer (n = 2, 0); (*l*) liomyosarcoma (n = 2, 0); (*m*) thyroid cancer (n = 1, 0); (*n*) chronic lymphocytic leukemia (n = 1, 0); (*o*) bladder cancer (n = 1, 0); (*p*) renal cell carcinoma (n = 1, 0); (*q*) periurethral cancer (n = 0, 1); and (*r*) seminoma (n = 0, 1; Table 1). The average patient age was 61 years (range: 23–85). Patients' performance status evaluated by Eastern Cooperative Oncology Group criteria was 0 (n = 68), 1 (n = 32), and 2 (n = 13). All of the patients showed failure to respond to chemotherapy, hormonal therapy, and/or radiotherapy with clinical stage IV or recurrence. Details regarding the characteristics of patients with each respective type of cancer were described in the other studies (21–24), currently in press (25) or under submission.

Vaccinated Peptides and Immune Responses. The number of patients receiving four, three, two, or one peptide without immediate type hypersensitivity were 50, 19, 8, or 1 in HLA-A24$^+$ patients, respectively, and 21, 9, 3, or 2 in HLA-A2$^+$ patients, respectively. CTL precursors reacting to peptides were detected in prevaccination PBMCs for vaccination; the frequency of vaccinated peptides is given in Table 2. The most frequently used peptide was the SART3$_{109}$ (38 of 78 cases), followed by the lck$_{208}$ (31 cases) in HLA-A24$^+$ patients. In HLA-A2$^+$ patients, the most frequently used peptide was the MAP$_{294}$ (15 of 35 cases), followed by the MAP$_{432}$ (14 cases). CTL activity was evaluated in postvaccination (sixth) PBMC to evaluate cellular immune responses to the vaccinated peptides. Increased cellular responses were most frequently observed when the SART3$_{315}$ peptide was vaccinated (9 of 22 cases, 41%) followed by the SART3$_{109}$ peptide (12 of 31 cases, 39%). Detailed results for each case have been reported elsewhere (21–25) and are summarized in Table 2. CTL activity to HLA-class I-restricted tumor cells was measured by the standard ^{51}Cr-release assay in pre and postvaccination (third and sixth) PBMCs. Sixteen of 76 cases tested (21%) showed increased HLA-class I-restricted cytotoxicity. DTH response at the site of a skin test during the first to sixth vaccination was most frequently observed when the SART3$_{315}$ peptide was vaccinated (9 of 26 cases of HLA-A24$^+$ patients). DTH response was most frequently observed in HLA-A2$^+$ patients vaccinated with the lck$_{422}$ peptide (6 of 12 cases), a summary of which is presented in Table 2. Humoral immune responses to the vaccinated peptides were simultaneously measured in both pre and postvaccination (third and sixth) sera. Increased levels of IgG antibodies reactive to peptides were most frequently observed when the SART3$_{109}$ was vaccinated (19 of 37 cases), as summarized in Table 2. It is of note that the UBE$_{43}$ peptide induced humoral immune responses in all five cases tested. Detailed results and the criteria of increased immune responses for each case have been reported elsewhere (21–25) and are under submission as separate studies.

Clinical Responses and Prognostic Marker Analysis. Of 113 cases, 5 cases showed partial response, 2 cases showed minor response, and the remaining 106 cases showed progressive disease. A median survival time of 113 cases was 346 ± 64.9 (±SE) days, and a 1-year survival rate was 44.6% (Fig. 1*A*). Twenty-two cases could not achieve one cycle of vaccination (six times) because of the rapid progression of tumors, whereas the remaining 91 cases received more than six vaccinations. The median survival time and 1-year survival rate of these 91 cases were 409 ± 15 days and 54.4%, respectively (Fig. 1*B*). In patients undergoing more than six vaccinations (n = 91), 60 cases had detectably increased levels of peptide-specific IgG antibody in their postvaccination sera against at least one peptide of at maximum four vaccination peptides, whereas the remaining 31 cases did not (Fig. 1*C*). Forty-two among 90 cases tested showed increased CTL activity response to at least one peptide of at maximum four vaccination peptides (Fig. 1*D*), and 16 of 73 cases tested showed increased CTL activity of HLA-class I-restricted cytotoxicity against tumor

Table 2 Vaccinated peptide and immune responses

Peptide name	Sequence	No. of vaccinated patients	Increased immune reactions to peptide[a] CTL activity	IgG antibody	DTH[b] response
HLA-A24-binding					
SART1 690	EYRGFTQDF	20	1 /17 (6%)	2 /19 (11%)	2 /20 (10%)
SART2 93	DYSARWNEI	19	3 /15 (20%)	4 /17 (24%)	0 /19
SART2 161	AYDFLYNYL	21	2 /16 (13%)	1 /18 (6%)	2 /21 (10%)
SART2 899	SYTRLFLIL	13	0 /7	2 /11 (18%)	3 /13 (23%)
SART3 109	VYDYNCHVDL	38	12 /31 (39%)	19 /37 (51%)	4 /38 (11%)
SART3 315	AYIDFEMKI	26	9 /22 (41%)	6 /25 (24%)	9 /26 (35%)
CypB84	KFHRVIKDF	4	0 /3	2 /3 (67%)	0 /4
CypB91	DFMIQGGDF	19	0 /17	1 /17 (6%)	1 /19 (5%)
lck208	HYTNASDGL	31	7 /25 (28%)	9 /29 (31%)	6 /31 (19%)
lck486	TFDYLRSVL	25	4 /21 (19%)	7 /22 (32%)	6 /25 (24%)
lck488	DYLRSVLEDF	26	3 /21 (14%)	1 /24 (4%)	5 /26 (19%)
ART1 170	EYCLKFTKL	9	0 /7	2 /7 (29%)	0 /9
ART4 13	AFLRHAAL	4	0 /1	0 /2	0 /4
ART4 75	DYPSLSATDI	19	1 /16 (6%)	0 /15	1 /19 (5%)
HLA-A02-binding					
SART3 302	LLQAEAPRL	6	0 /5	0 /5	0 /6
SART3 309	RLAEYQAYI	9	0 /7	1 /7 (14%)	0 /9
CypB129	KLKHYGPGWV	6	0 /6	2 /6 (33%)	1 /6 (17%)
CypB172	VLEGMEVV	6	1 /4 (25%)	3 /5 (60%)	1 /6 (17%)
lck246	KLVERLGAA	7	2 /7 (29%)	6 /7 (86%)	0 /7
lck422	DVWSFGILL	12	3 /11 (27%)	0 /10	6 /12 (50%)
MAP294	GLLFLHTRT	15	1 /13 (8%)	8 /15 (53%)	7 /15 (47%)
MAP432	DLLSHAFFA	14	3 /11 (27%)	5 /13 (38%)	4 /14 (29%)
WHSC103	ASLDSDPWV	8	0 /6	1 /8 (13%)	1 /8 (13%)
WHSC141	ILGELREKV	8	1 /4 (25%)	2 /5 (40%)	0 /8
UBE43	RLQEWCSVI	6	1 /5 (20%)	5 /5 (100%)	2 /6 (33%)
UBE85	LIADFLSGL	2	1 /2 (50%)	1 /2 (50%)	0 /2
UBE208	ILPRKHHRI	1	0 /1	1 /1 (100%)	0 /1
HNRPL140	ALVEFEDVL	5	0 /5	0 /5	0 /5
HNRPL501	NVLHFFNAPL	13	1 /11 (9%)	3 /13 (23%)	2 /13 (15%)
EIF51	RIIYDRKFL	1	0 /1	1 /1 (100%)	0 /1

[a] No. of tested cases and % positive in parentheses are shown.
[b] DTH, delayed-type hypersensitivity.

cells (Fig. 1*E*) in their postvaccination PBMCs. Thirty-four of 91 cases showed DTH response to at least one peptide among at maximum four vaccination peptides until the sixth vaccination (Fig. 1*F*). None of the cellular responses (peptide-specific CTL precursors, tumor-reactive CTL activity, or DTH reaction) correlated with overall survival. In contrast, the overall survival of patients whose sera showed increased levels of peptide-reactive IgG antibodies ($n = 60$) was more significantly prolonged ($P = 0.0003$) than that of patients whose sera did not show such increased levels ($n = 31$). In addition, multivariate analysis was carried out with factors of age, gender, performance status, HLA typing, increased peptide-reactive IgG antibodies levels, increased CTL responses, increased cytotoxicity, and observed DTH reaction. Among them, significantly contributed factors for overall survival of 91 cases were increased peptide-reactive IgG antibodies levels ($P = 0.0014$) and performance status 0 or 1 ($P = 0.0046$), although only 4 of 91 cases were performance status 2.

Detailed Analysis of Antibody Responses and Survival Time. To obtain a better understanding of the relationship between antibody response and survival time, representative results of serial measurements of IgG reactive to the peptides for ≤12 vaccinations in the patients whose sera showed the positive responses to the vaccinated peptides were shown in Fig. 2. The result on one peptide per patient was given if a serum reacted to several peptides to save space on the study. The data showed in the other studies that were cited in this one (21–25) or those under submission were not given to avoid double publication. Positive responses were judged by the two criteria. The first criterion is the case if the prevaccination serum showed no reactions, and the postvaccination serum showed at least significant levels of IgG (>0.02 of net OD value at a serum dilution of 1:100) specific to the vaccinated peptides. This case was observed in 50 of 60 patients tested. Some of the cases are shown in Fig. 2. The other criterion is the case if the postvaccination serum showed ≥2-fold increase of the IgG level at a serum dilution of 1:100 than that of the prevaccination serum. This case was observed in the remaining 10 cases. Some of these cases are shown in Fig. 2. There was, however, no apparent difference of the survival time between the two groups (Fig. 3*A*).

In regard to the kinetics, positive antibody responses were induced in sera after the third vaccinations in 19 cases and after the sixth vaccinations in the remaining 41 cases, respectively. There was, however, no apparent difference of the survival time between the two groups (Fig. 3*B*). Among 113 cases shown in