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-

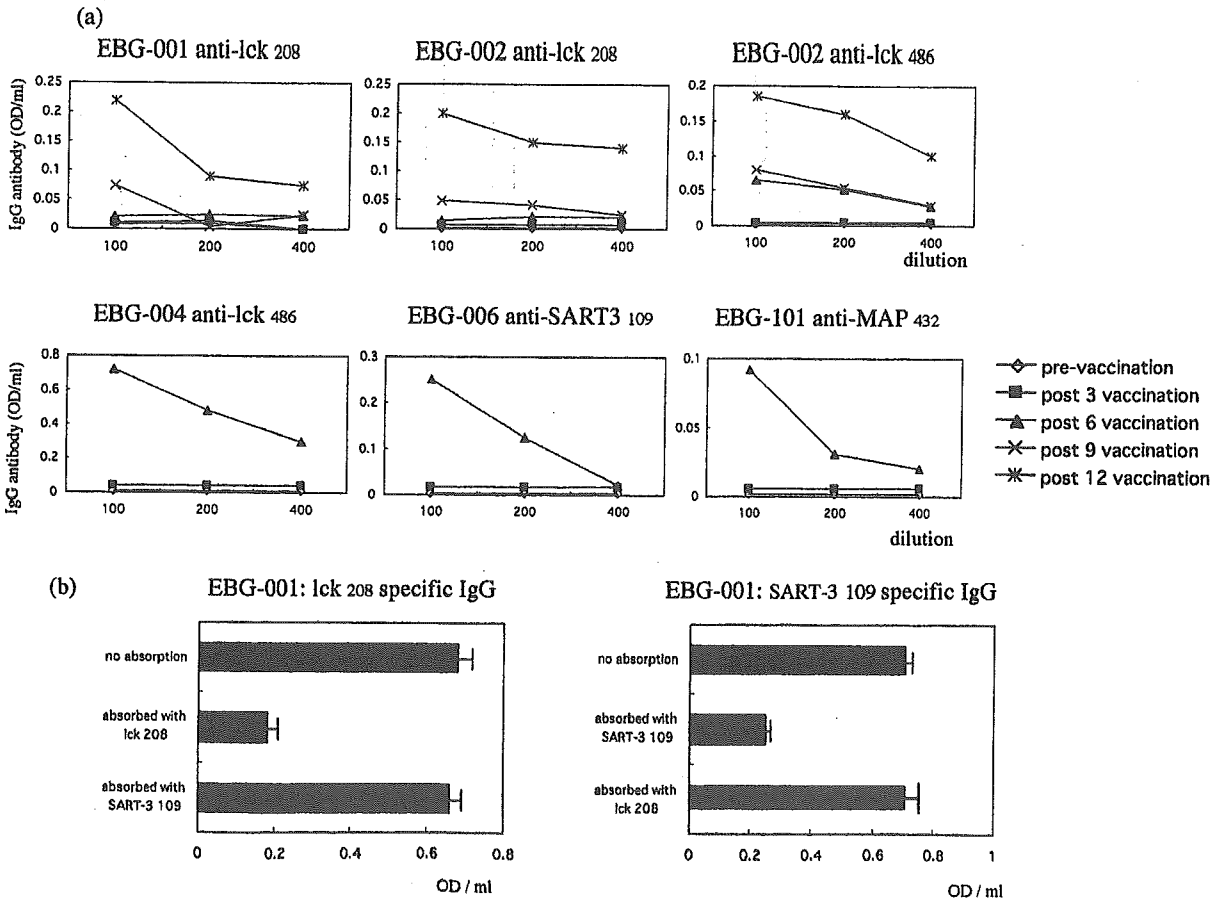
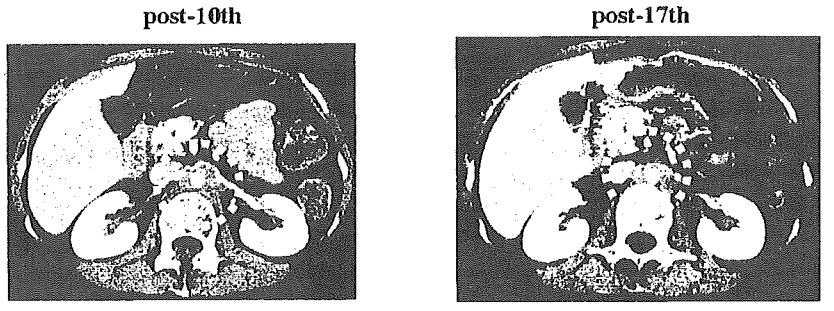
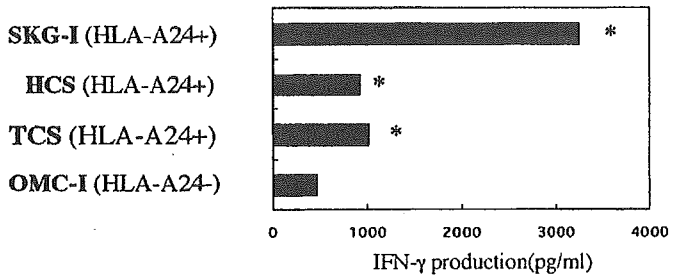


FIGURE 3. Serum IgG reactive to vaccinated peptides. **a:** The levels of peptide-specific IgG of pre- and post-vaccination plasma from 5 patients were determined by ELISA. **b:** Plasma from patient EBG-001 after the 12th peptide vaccination was cultured with the indicated peptide-coated plates. Thereafter,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

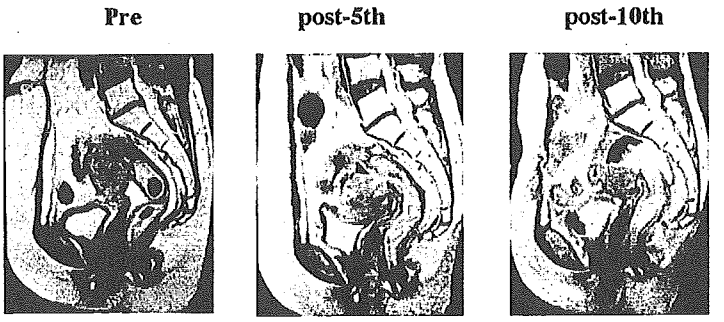
(a) [EBG-001]



Shrinkage of tumor 42%
Level of CEA(ng/ml) 188 105

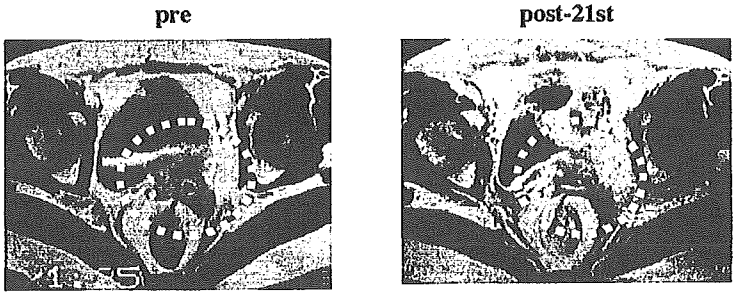


(b) [EBG-101]



Shrinkage of tumor 26% 48%
Level of SCC (ng/ml) 273 289 36

(c) [EBG-103]



Shrinkage of tumor 13%

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.

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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Co-expression of Y box-binding protein-1 and P-glycoprotein as a prognostic marker for survival in epithelial ovarian cancer

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Abstract

Objectives. This study aimed to observe the expressions of Y box-binding protein-1 (YB-1) and P-glycoprotein (P-gp) in primary ovarian tumor and to determine whether they act as biomarkers for survival in epithelial ovarian cancer.

Methods. The expressions of YB-1 and P-gp were examined immunohistochemically in 59 patients who were treated from 1997 to 2000 at Kurume University Hospital. Samples were paraffin-embedded primary ovarian cancer tissue taken from the surgical specimens.

Results. Of the 59 primary ovarian tumors examined, 32 (54.2%) and 18 (30.5%) were positive for YB-1 and P-gp, respectively. Co-expression of these two proteins was observed in 20.3% (12/59) cases. Patients showing this co-expression had a worse 3-year survival than those without co-expression (40.0% vs. 73.1%, $P = 0.0447$). This co-expression significantly correlated with poor prognosis according to multivariate analysis ($P = 0.0007$).

Conclusion. Co-expression of YB-1 and P-gp emerged as a promising relevant biomarker for unfavorable prognosis in ovarian cancer.

Keywords: Ovarian cancer; Y box-binding protein-1; P-glycoprotein; Multidrug resistance; Prognosis

In the Western world, ovarian cancer is the leading cause of death from gynecologic malignancies. According to the *Cancer Facts and Figures 2003* from American Cancer Society, it caused 26,800 female deaths in the United States. One of the reasons for this high mortality rate could be the appearance of resistance against anticancer drugs. Approximately 20% of ovarian cancers are considered to be intrinsically resistant to first-line chemotherapy [1]. Therefore, overcoming drug resistance could be the key for developing a more effective chemotherapy for ovarian cancer. The expression of various proteins could function to express an intrinsic resistance to chemotherapy [2]. The overexpression of P-glycoprotein (P-gp) has been found in many chemoresistant tumors. This protein encoded by the MDR1 gene, functions as efflux pump, and reduces the accumulation of anticancer drugs in tumor cell [3].

Recently, it was reported that the Y-box, an inverted CCAAT box, was located on the promoter area of MDR1 genes, and that Y box-binding protein-1 (YB-1), a member of the DNA binding protein family, was associated with expression of drug resistance in human tumors [4–7]. In a clinical study, patients with YB-1 nuclear-positive tumors had a poor prognosis when compared with those with YB-1 nuclear-negative tumors in ovarian serous adenocarcinoma [8]. These results suggest the presence of intrinsic drug resistance expressed by YB-1 protein. However, it remained unclear if YB-1 would play an important role in ovarian carcinomas, other than serous adenocarcinoma.

Therefore, the aim of this study was (1) to examine the expression of YB-1 and its target genes such as P-gp in all types of primary epithelial ovarian carcinoma, and (2) to investigate a relation between expression of these proteins and clinicopathologic factors as well as survival in epithelial ovarian cancer.

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Materials and methods

Patients

From 1997 to 2000, 59 patients with histologically proven ovarian cancer were treated at the Department of Obstetrics and Gynecology, Kurume University Hospital, Japan. All patients received primary surgery and adjuvant chemotherapy. The following data were collected from their medical records, including age, clinical stage, histological subtypes and grade, size of residual tumor at the primary surgery, and regimens of postsurgical chemotherapy. Histological grading was determined according to The International Federation of Gynecology and Obstetrics (FIGO, 1988) grading system. The patients' characteristics are presented in Table 1.

Primary antibody

Polyclonal anti-YBC antibody to YB-1 was generated as described previously [4]. Briefly, a synthetic peptide composed of 15 amino acids (residues 299–313) in the tail domain of the YB-1 protein was used to generate antibodies in a rabbit. The rabbit polyclonal antibody induced by this antigen was affinity-purified on columns prepared from the same peptide. YB-1 was diluted in PBS at a dilution of 1:5000 for immunohistochemical staining. JSB-1 antibody (1:20) monoclonal antibody directed against P-gp was obtained from Nichirei (Japan).

Table 1
Patient characteristics

Number of patients	59
Age: mean ± SD (years)	51.4 ± 14.6
Clinical stage (FIGO)	No. (%)
I	24 (40.6)
II	5 (8.5)
III	25 (42.4)
IV	5 (8.5)
Histology	
Serous	18 (30.5)
Mucinous	19 (32.2)
Endometrioid	9 (15.3)
Clear cell	8 (13.5)
Others	5 (8.5)
Grading	
Grade 1	17 (28.8)
Grade 2	20 (33.9)
Grade 3	22 (37.3)
Residual tumor after first surgery	
No	27 (45.8)
Yes	32 (54.2)
Chemotherapy	
CP/CAP[a]	19 (32.2)
CDDP/CBDCA + TAXOL	21 (35.6)
CDDP + CPT-11	19 (32.2)

[a] C: cyclophosphamide; P: cisplatin; A: adriamycin.

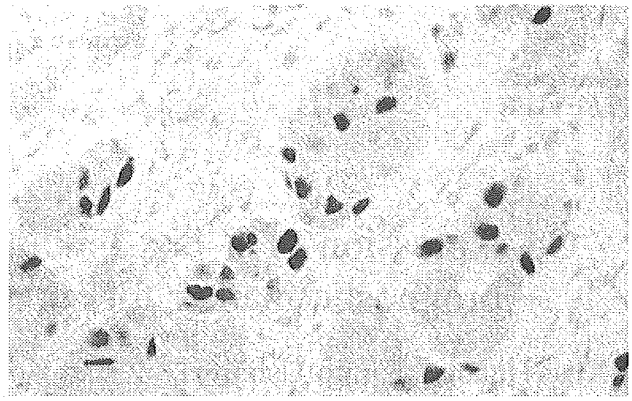

Fig. 1. Immunohistochemical YB-1 staining of ovarian cancer. The nuclei were strongly stained and cytoplasm was weakly stained (original magnification ×400).

Immunohistochemical staining

A formalin-fixed, paraffin-embedded, 4-μm section was obtained from each sample of primary epithelial ovarian cancer. Immunohistochemical (IHC) staining was performed with a streptavidin–biotin–peroxidase complex method. Samples were deparaffinized in xylene and dehydrated in a graded series of alcohol. To recover the antigenicity of YB-1, the sections were immersed in a citrate buffer and pretreated for 15 min at 120°C by an autoclave, and thereafter exposed to the primary antibody at room temperature for 60 min. A subsequent reaction was made using a streptavidin–biotin–peroxidase kit (Nichirei). For the detection of P-gp, the sections were exposed at room temperature for 15 min and a catalyzed signal amplification kit (CSA kit, DAKO) was used. The sections were stained with freshly prepared diaminobenzidine solution and then counterstained with hematoxylin.

The cells with strongly stained nuclei and weakly stained cytoplasm were interpreted as positive cells for YB-1. We defined those only with weakly stained cytoplasm as negative for YB-1. Either cytoplasm or nuclear staining was

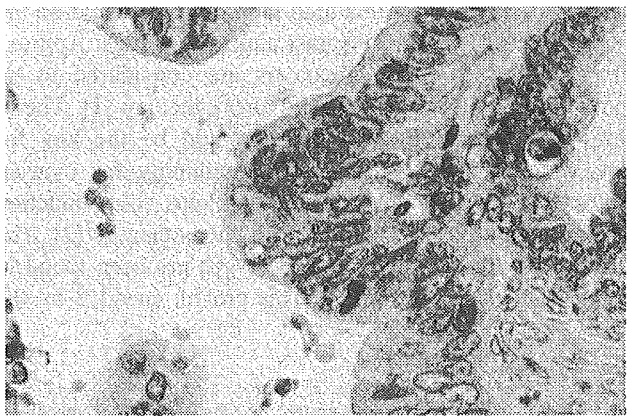

Fig. 2. Immunohistochemical P-gp staining of ovarian cancer. The cytoplasm was stained (original magnification ×400).

Table 2
Clinical-pathologic features and IHC data for 59 cases of ovarian cancer

Prognostic factors	YB-1 expression			P-gp expression		
	Negative	Positive	P^a	Negative	Positive	P^a
FIGO stage						
Stage I + II	15 (51.7.)	14 (48.3)	0.366	18 (62.1)	11 (37.9)	0.223
Stage III + IV	12 (40.0)	18 (60.0)		23 (76.7)	7 (23.3)	
Histology						
Serous	7 (38.9)	11 (61.1)		10 (55.6)	8 (44.4)	
Mucinous	11 (57.9)	8 (42.1)	0.513	15 (78.9)	4 (21.1)	0.184
Endometriod	4 (44.4)	5 (55.6)		8 (88.9)	1 (11.1)	
Clear cell	2 (25.0)	6 (75.0)		6 (75.0)	2 (25.0)	
Others	3 (60.0)	2 (40.0)		2 (40.0)	3 (60.0)	
Grade						
1	10 (58.8)	7 (41.2)	0.07	13 (76.5)	4 (23.5)	0.223
2	5 (25.0)	15 (75.0)		11 (55.0)	9 (45.0)	
3	12 (54.5)	10 (45.5)		17 (77.3)	6 (22.7)	
Residual						
No	13 (48.1)	14 (51.9)	0.735	19 (70.4)	8 (29.6)	0.893
Yes	14 (43.7)	18 (56.3)		22 (68.8)	10 (31.2)	
Total	27 (45.8)	32 (54.2)		41 (69.5)	18 (30.5)	

[a] Chi-square test.

regarded as positive for P-gp. Both the positive controls of YB-1 and P-gp were samples from the colorectal cancer.

Statistical analysis

The SPSS for Windows 10.0 computer program was used for statistical analysis. The statistical significance between clinical and pathological characteristics was evaluated using the chi-square test. The Spearman rank correlation test was used to determine the correlation between two variables. The survival distribution was calculated using the Kaplan–Meier method, and survival curves were compared using log-rank statistics. All variables that were significant at the 5% level in the univariate analysis were included in a Cox proportional hazard regression model. Model selection for identifying the variables having important effects on survival was based on a forward stepwise procedure. All tests were performed at a significance level of $\alpha = 0.05$.

Results

All 59 ovarian carcinoma showed positive immunoreaction for anti-YBC in the cytoplasm. In 32 of 59 cases (54.2%), YB-1 was expressed in the nucleus (Fig. 1). P-gp expression was found in 30.5% (18/59) ovarian cancers (Fig. 2). There was no significant correlation between the expression of YB-1 or P-gp and clinicopathologic factors, such as clinical stage, presence of residual tumor, type of histology, and tumor grade (Table 2).

Table 3
Type of expressions of YB-1 and P-gp ($n = 59$)

	YB-1(−)	YB-1(+)
P-gp(−)	21(35.6%)	20(33.9%)
P-gp(+)	6(10.2%)	12(20.3%)

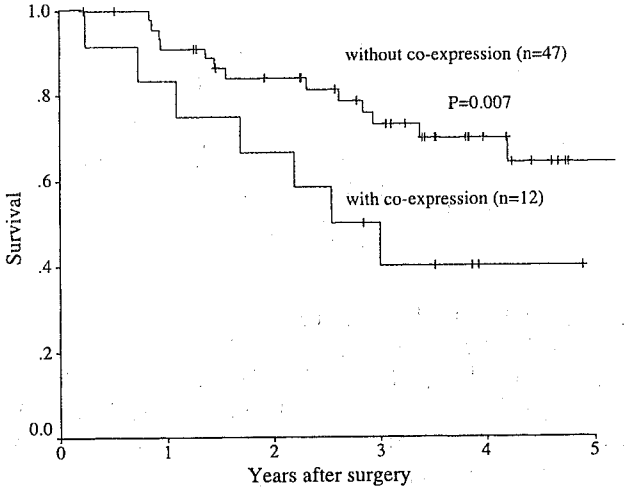

Fig. 3. Kaplan–Meier survival probability stratified by co-expression of YB-1 and P-gp.

Neither YB-1 nor P-gp expression was found in 21/59 (35.6%) of cases. In 26/59 (44.1%) patients, either YB-1 or P-gp expression was found. The co-expression of both factors was recognized in the same specimens from 12/59 (20.3%) patients (Table 3). There was no relationship between the expression of YB-1 and that of P-gp ($P = 0.204$).

No significant difference in overall survival was seen between the patients with YB-1 or P-gp expression and those without either expression. However, the patients with co-expression of YB-1 and P-gp showed lower 1-, 2-, and 3-

Table 4
Univariate survival analysis by Kaplan–Meier

	Factors	Total	Dead cases	P value
YB-1	(−)	27	5	0.0851
	(+)	32	16	
P-gp	(−)	41	14	0.5034
	(+)	18	7	
Co-expression	no	47	14	0.0447
	yes	12	7	
Stage	I + II	29	4	0.0028
	III + IV	30	17	
Histology	serous	18	5	0.5894
	mucinous	19	8	
	others	22	8	
Grade	1	17	3	0.2795
	2	20	9	
	3	22	9	
Residual	no	27	5	0.0034
	yes	32	16	
Distant metastasis	no	51	14	0.0029
	yes	8	7	
Recurrence	no	34	4	0.0000
	yes	25	17	

Table 5
Prognostic factors for overall survival by Cox regression analysis

Variable	*B*	SE	Wald	RR	95% CI for RR		*P* value
					Lower	Upper	
YB-1 + P-gp	1.379	0.514	7.200	3.972	1.450	10.878	0.007
Stage	1.265	0.601	4.429	3.542	1.091	11.505	0.035
Recurrence	1.670	0.580	8.279	5.312	1.703	16.571	0.004

YB-1 + P-gp: co-expression of YB-1 and P-gp.

year survival rates than did patients without co-expression (91.1%, 84.0%, 73.1% vs. 83.3%, 66.7%, 40.0%, respectively) (Fig. 3). Besides co-expression of YB-1 and P-gp, stage, residual presence at primary surgery, distant metastasis, and recurrence also were significantly different according to univariate analysis (Table 4).

Five variables that were significant at the 5% level in the univariate analysis were entered into a multivariate analysis model (Table 5). Co-expressions of YB-1 and P-gp, as well as stage and recurrence, were three significant independent prognostic factors.

Discussion

In the present study, 52% of ovarian cancer patients expressed YB-1 in the nucleus of the cancer cell. This was slightly higher than the results reported by other studies including 30% in serous carcinoma [8] and 45.7% in all other types of all ovarian cancer [9]. There was no significant relationship between YB-1 expression and any clinicopathologic prognostic factors, such as clinical stage, histological types or grade, and residuals after first surgery. The same results were also observed in ovarian serous carcinoma [8] and osteosarcoma [6]. In the present study, no relationship was seen between patient survival and YB-1 expression. These results disagreed with the report of stage III ovarian serous carcinoma [8]. We had only seven cases with stage III serous ovarian cancer in our series, including two patients with negative nuclear YB-1 that showed relatively longer survival (3.39 and 5.68 years, respectively). Both of them were also P-gp negative. We were unable to prove the significance between YB-1 and prognosis in stage III serous carcinoma in our series because of the limitation of cases. In breast cancer and colorectal carcinoma, no relationship between YB-1 expression and survival was observed [7,10].

The overexpression of P-gp appears to be closely associated with multidrug resistance in human cancers [11,12]. In ovarian tumors, the frequency of P-gp expression varied over a wide range (7–80%) [3,8,13–15]. A higher detectable ratio of P-gp expression was reported with amplification of MDR1 gene using the polymerase chain reaction (PCR) method than using the IHC method because of the higher sensitivity in PCR method [15]. We found P-gp expression in 30.5% of our untreated ovarian cancers. Review of the literatures suggests that our result was close to values reported for P-gp in the literatures, which were also using IHC in untreated ovarian cancer [16–18]. Nevertheless, no relationship had been found between survival and P-gp expression in our paper and the literature regarding primary ovarian tumors [3,13,19].

It was reported that overexpression of P-gp was linked to YB-1 expression because MDR-1 which encodes P-gp is activated by nuclear localization of YB-1 [4,20]. Correlation between overexpression of nuclear YB-1 and P-gp was reported in osteosarcoma and breast cancer [6,7]. But there was no correlation in ovarian cancer or lung cancer [8,21]. This is probably due to tissue-specific characteristics of lung and ovarian cancer. On the other hand, in the present study, the co-expression of YB-1 and P-gp significantly correlated with patient survival. Moreover, the co-expression of YB-1 and P-gp was shown to be an independent prognostic factor by both univariate and multivariate analysis as were clinical stage and history of recurrence. Many parameters are involved in the mechanism of drug resistance. Although this mechanism is complicated, the simultaneous appearance of multiple factors of drug resistance would be a further unfavorable combination. YB-1 is translocated from cytoplasm into the nucleus of tumor cells by exogenous stress such as ultraviolet irradiation or cytotoxic drugs [22]. Some tumors would acquire transient drug resistance after chemotherapy. In the present study, the analyzed drug resistance was only an intrinsic, not an acquired one, because all our samples were taken at primary surgery without having undergone chemotherapy. For the next step, tissue sampling from various points during treatment, such as at second-look operation, secondary cytoreductive surgery, or surgery for recurrent tumor, should be done. The change of drug resistance during chemotherapy may be detected from those samples.

In conclusion, co-expression of YB-1 and P-gp could be a biomarker predicting poor prognosis in ovarian cancer. Further studies are now in progress, which will help in choosing the most appropriate drugs for patients with persistent or recurrent tumor.

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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.

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

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.

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

	Regimen 1					Regimen 2				
Toxicity	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.

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)	–	–	
	SARTI 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.

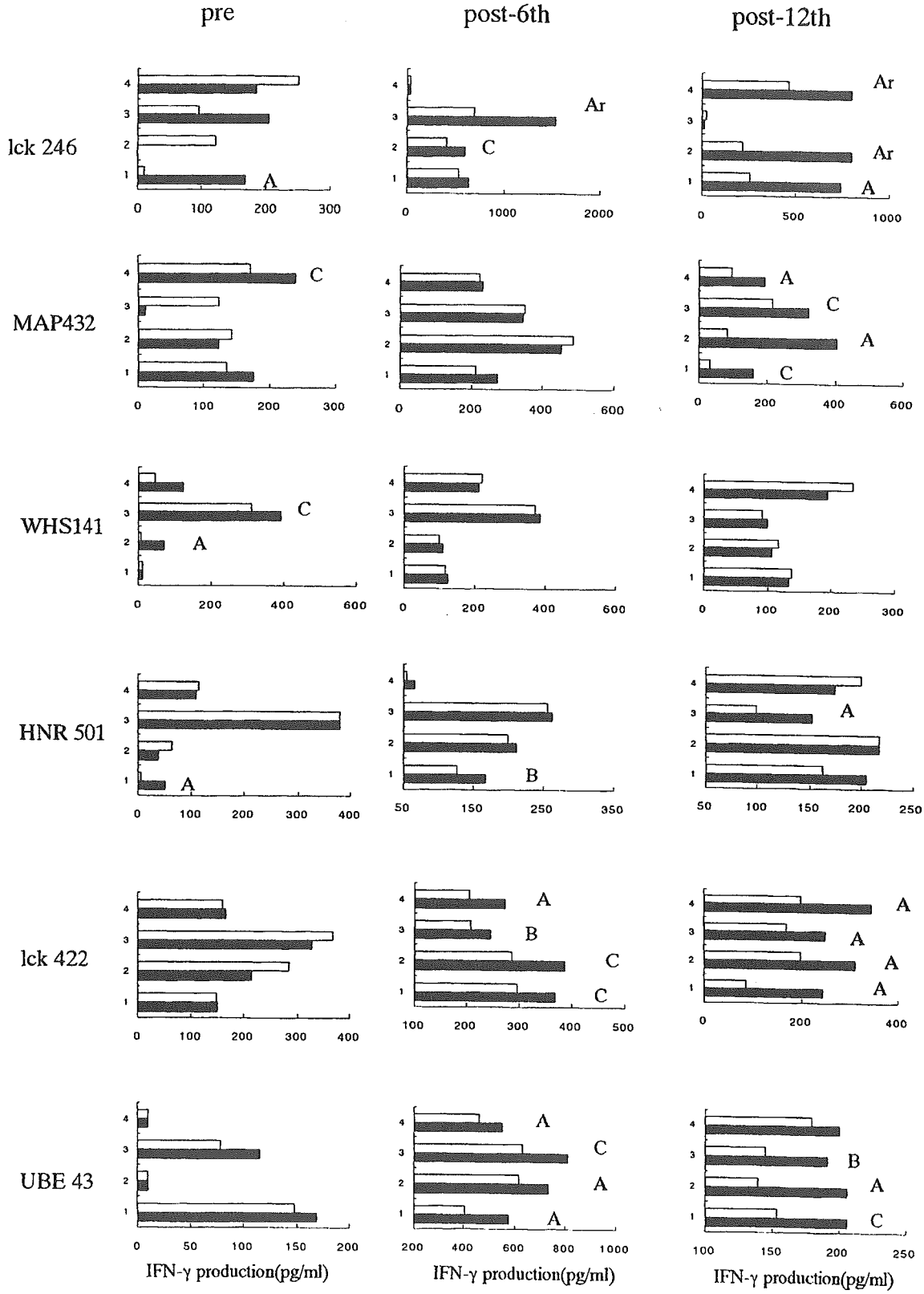

FIGURE 1. Detection of peptide-specific CTL precursors. Pre- and post- (6^{th} and 12^{th}) vaccination PBMCs from patient EBG-103 (HLA-$A2^{+}$) were applied for the screening of peptide-specific CTL precursors. Values represent IFN-γ production by the in vitro cultured PBMCs. Criteria for evaluation 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.