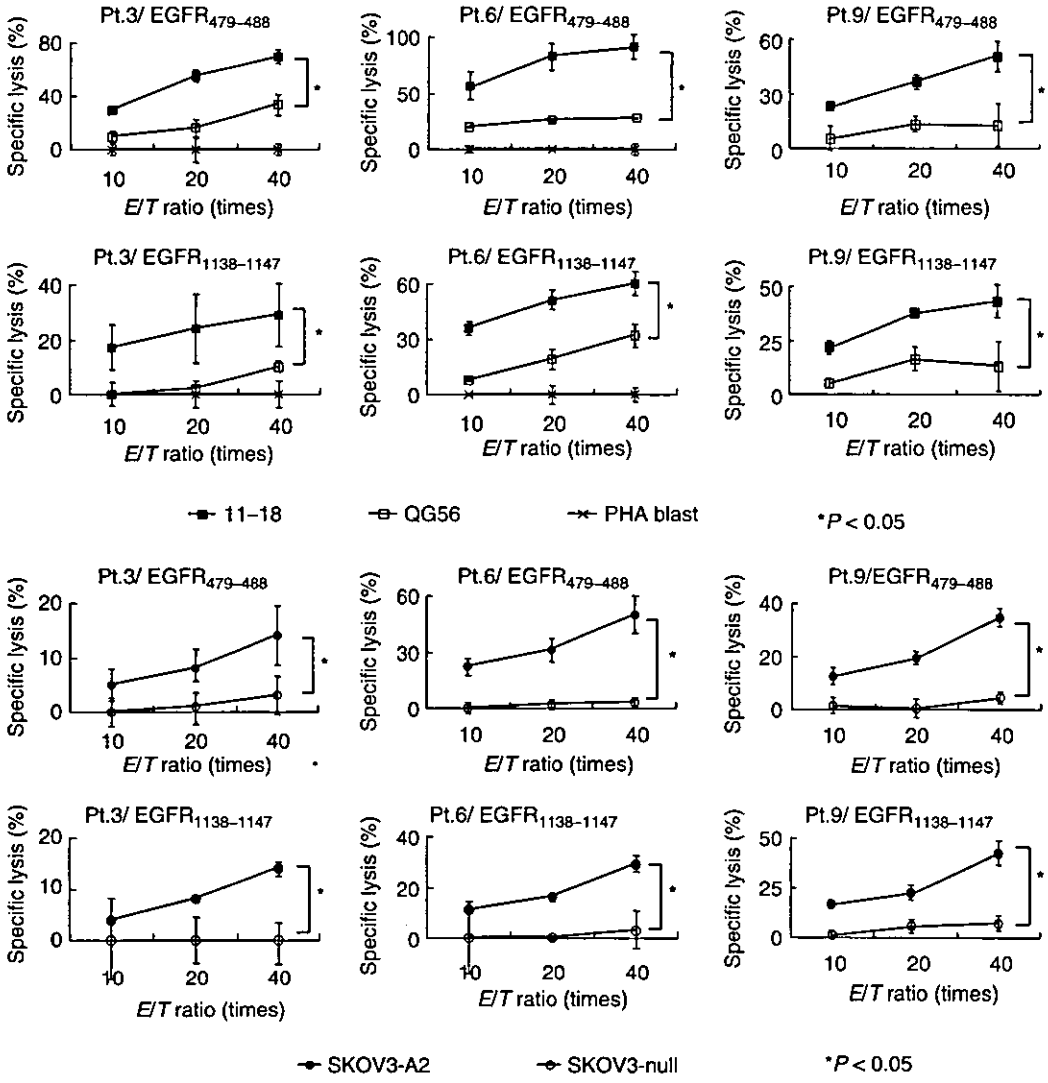

Figure 5 Cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against the following cancer cell lines: 11–18 (HLA-A2$^+$, EGFR$^+$), QG56 (HLA-A2$^-$, EGFR$^+$), SKOV3-A2 (HLA-A2$^+$, EGFR$^+$), and SKOV3 (HLA-A2$^-$, EGFR$^+$). PHA-blastoid T cells (HLA-A2$^+$, EGFR$^-$) were also used as a negative control. The standard 6-h ^{51}Cr-release assay was performed at three *E/T* (effector to target) ratios. The representative results of three cancer patients (Pts.3, 6, and 9) are shown in the figure. Values represent the mean ± s.d. of % specific lysis. An asterisk (*) indicates $P<0.05$ by a two-tailed Student's *t*-test.

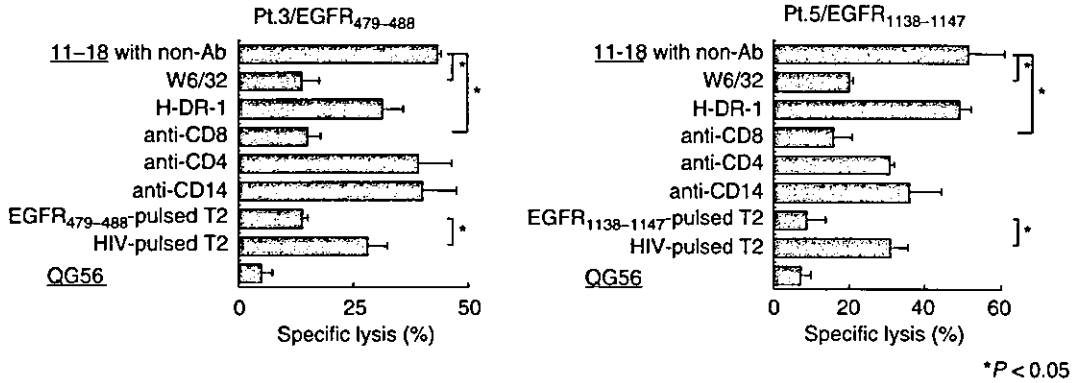

Figure 6 Inhibition and competition assays. Peptide-stimulated PBMCs were tested for their restriction and peptide-specificity of cytotoxicity against 11–18 (HLA-A2$^+$, EGFR$^+$) and QG56 (HLA-A2$^-$, EGFR$^+$) by the standard 6-h ^{51}Cr-release assay. In all, 20 μg/ml of anti-HLA-class I (W6/32, IgG2a), anti-HLA-class II (H-DR-1, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a), and anti-CD4 (Nu-Th/i, IgG1) mAb were used for the inhibition assays. Anti-CD14 (JML-H14, IgG2a) mAb served as a negative control. For the competition assay, unlabelled T2 cells pulsed with the corresponding peptide or an HIV peptide as a negative control were added to the ^{51}Cr-release assay at a cold-to-hot target cell ratio of 10:1. The 6-h ^{51}Cr-release assay was performed at an *E/T* ratio of 10:1. An asterisk (*) indicates $P<0.05$ by a two-tailed Student's *t*-test. Values represent the mean ± s.d. of % specific lysis.

are also presently unclear. Those antipeptide IgGs, however, have not been found to react to the mother proteins to the degree that they have been tested, which is in agreement with the present finding of a lack of anti-EGFR-derived-peptide IgG reactivity in response to the EGFR protein shown. Sera possessing anti-EGFR-derived-peptide IgGs also failed to show either direct growth inhibition of tumour cells *in vitro* or to elicit antibody-dependent cell-mediated cytotoxicity to tumour cells to the degree that they have been tested. Therefore, anti-EGFR-derived-peptide IgGs may not act directly on tumour cells.

These antipeptide IgGs did not react to the mother protein, and also failed to show either the direct inhibition of tumour cell growth *in vitro* or to elicit antibody-dependent cell-mediated cytotoxicity to tumour cells as far as tested. It is well known that T cells in the circulation rarely infiltrate into tumour sites. In contrast, IgG molecules might easily reach either peritumour sites or intratumour sites, which in turn facilitate inflammatory reactions at the tumour cites. This assumption is in part supported by the recent observation that significant levels of inflammatory responses were observed around prostate cancers at the time of surgery in patients who received peptide vaccinations based on information regarding antibodies reactive to peptides before radical prostatectomy (Noguchi *et al* unpublished results). We also reported that IgG reactive to these CTL-epitope peptides are either lacking or unbalanced in the sera of patients with atopic disease (Kawamoto *et al*, 2003). The results shown in this study along with those from noncancerous subjects suggests that these peptide-reactive IgGs play a role in host-defence against various diseases, although further studies are needed to clarify their biological role as well as their mechanism of action.

Although further studies are needed to clarify the biological role as well as the mechanism of action of antipeptide antibodies, the two peptides recognised by both cellular and humoral immune responses can be presumed to be more immunogenic than those recognised by the cellular response alone. The HLA-A2 allele is found in 40% of Japanese, 50% of Caucasians, and 16% of Africans. There are several major subtypes of the HLA-A2 allele. The frequencies of HLA-A0201, -A0206, and -A0207 among HLA-A2-positive Japanese are about 45, 36, and 17%, respectively, whereas HLA-A0201 is the predominant subtype among HLA-A2-positive Western Caucasians (96%), African Blacks (62%), and Sardinian Caucasians (59%) (Imanishi *et al*, 1992). These two peptides at positions 479–488 and 1138–1147 possessed the ability to induce HLA-A2-restricted and tumour-specific CTLs from PBMCs of cancer patients with at least the three different HLA-A2 subtypes shown above.

Epidermal growth factor receptor is highly expressed in a number of human tumours (Coussens *et al*, 1985; Yamamoto *et al*, 1986; Salomon *et al*, 1995), and many clinical trials of EGFR-targeted therapies have been going on. In those clinical trials, various toxicities (mainly, acneiform rash and diarrhoea) were reported although the frequency and severity of these adverse events were relatively low (Dittrich *et al*, 2002; Herbst *et al*, 2002; Mendelsohn and Baselga, 2003). We have used self-antigen-derived peptides, such as SART1, SART3, and lck, in phase I clinical studies of individualised peptide vaccination for far advanced cancer patients. In these clinical studies, no severe adverse events, except for local redness and swelling of injection site, were observed (Mine *et al*, 2003; Noguchi *et al*, 2003; Sato *et al*, 2003; Tsuda *et al*, 2004). Epidermal growth factor receptor-derived peptides shown in this study are now under clinical trials in our hospitals as phase I study of individualised peptide vaccination for far advanced cancer patients, but the vaccination of these EGFR-peptides was not associated with acneiform rash and diarrhoea. However, careful observation throughout the phase I study is needed to obtain the safety of these peptides. The phase II study with these peptides is planned to see whether EGFR is one of attractive targets for immunotherapy for far advanced epithelial cancer patients or not.

In conclusion, these findings may provide new insight for the development of an EGFR-based immunotherapy beneficial for substantial numbers of epithelial cancer patients throughout the world.

ACKNOWLEDGEMENTS

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (no. 12213134 to KI), from the Japan Society for the Promotion of Science (no. 14570526 to SS), and from the Ministry of Health and Welfare of Japan (no. H14-trans-002, 11-16, and H12-cancer-004 to KI, and no. H14-cancer-033 to SS).

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Immunological Monitoring During Combination of Patient-Oriented Peptide Vaccination and Estramustine Phosphate in Patients With Metastatic Hormone Refractory Prostate Cancer

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BACKGROUND. Additive antitumor effects could be achieved by combination of immunotherapy and cytotoxic agents with no or minimum suppression.
METHODS. Thirteen patients positive for human leukocyte antigen (HLA)-A24 or -A2 with metastatic hormone refractory prostate cancer (HRPC) who had failed to respond to the prior-peptide vaccination were entered in the combined peptide vaccination and estramustine phosphate. Conducted immune monitoring on those 13 patients were mainly peptide-specific cytotoxic T lymphocyte (CTL) precursor analysis by IFN-γ productions and peptide-reactive IgG by an enzyme-linked immunosorbent assay (ELISA).
RESULTS. Grade 3 arrhythmia or cerebral infarction was observed in two cases, and Grade 1 or 2 dermatologic reaction at the vaccination sites was observed in all 13 cases. Eleven patients who received more than one cycle of treatment were eligible for immunological and clinical evaluation. There was no significant immunosuppression in most cases when the peptide and a half dose (280 mg/day) of estramustine were administrated, whereas severe immunosuppression was observed in the first two patients who received both the peptide and a full dose (560 mg/day) estramustine. Augmentation of peptide-specific CTL precursors or peptide-specific IgG was observed in 6 of 11 or 10 of 11 cases, respectively. Ten of 11 patients showed serum prostate-specific antigen (PSA) level decrease from the baseline including 8 patients with a serum PSA level decrease of ≥50%.
CONCLUSIONS. These results encouraged the further evaluation of the combination of peptide vaccination and low-dose estramustine phosphate for metastatic HRPC patients. *Prostate 60: 32–45, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; immunotherapy; cancer vaccine; estramustine phosphate**

INTRODUCTION

The optimal treatment strategy for patients with metastatic hormone-refractory prostate cancer (HRPC) continues to represent a challenge for oncologists. The median survival duration of patients with metastatic HRPC is about 12 months [1–3]. Although, chemotherapy with mitoxantrone offers a palliative benefit [1,2],

Abbreviations: CTL, cytotoxic T lymphocyte; PBMCs, peripheral blood mononuclear cells; HLA, human leukocyte antigen; HRPC, hormone refractory-prostate cancer; PSA, prostate-specific antigen; ELISA, enzyme-linked immunosorbent assay; DHT, delayed-type hypersensitivity; CT, computed tomography; CR, complete response; PR, partial response; PD, progression; Ar, armed response.

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Received 1 August 2003; Accepted 4 November 2003
DOI 10.1002/pros.20011
Published online 5 January 2004 in Wiley InterScience (www.interscience.wiley.com).

no treatment has been shown to prolong survival. Recently, Phase II trials of estramustine-based or taxane-based regimes reported a ≥50% decrease in levels of serum prostate-specific antigen (PSA) in 45–67% of patients [4–8]. However, these combinations were associated with a significant degree of nausea, diarrhea, leukopenia, and cumulative fluid retention and an increased risk for thrombotic events, precluding its use in patients with a poor performance status. In addition, none of these regimes is associated with prolonged survival and the number of patients in these studies was limited.

Many tumor antigens recognized by human leukocyte-associated antigens (HLA) Class I-restricted cytotoxic T lymphocytes (CTLs) have been identified in the past decade [9,10], and new approaches for HRPC with tumor vaccines have been investigated. Phase I/II clinical trials with dendritic cell-based immunotherapy have been conducted, and also a vaccine consisting of recombinant prostate-specific membrane antigen (PSMA) and adjuvant has been tested in prostate cancer patients [11,12]. Our approach in the immunotherapy for HRPC patients is a pre-vaccination measurement of peptide-specific CTL precursors in the circulation of cancer patients reactive to 30 kinds of vaccine candidates with the ability to induce CTLs, followed by administration of only reactive peptides (patient-oriented peptide vaccination) as reported previously [13,14]. We recently completed our Phase I clinical trial for HRPC to assess the safe administration of these peptides [15]. The adverse events of this immunotherapy were less severe than those of conventional therapies although the clinical responses of this trial have been limited. It is suggested that additive antitumor effects could be achieved by combination of peptide vaccination and cytotxic agents when the cytotoxic agents had minimum suppression of immune system.

Estramustine phosphate is a stable conjugate of estradiol and nitrogen mustard that possesses antimitotic properties and causes distruption of microtubule organization [16]. Estramustine phosphate has been subjected to many Phase II and III clinical trials in the last 25 years as a second-line treatment of HRPC in addition to primary treatment. The advantage of estramustine phosphate over other cytotoxic drugs is its ease of administration (oral) and relatively good tolerability at the effective dose.

The aim of this study was to evaluate the clinical and immunological responses of a combination of patient-oriented peptide vaccination and oral estramustine phosphate in patients with HRPC by analyzing serially measured serum PSA, bone turnover marker together with clinical bone scan recordings, peptide-specific CTL precursors by IFN-γ-release assay, and peptide-reactive IgG by an enzyme-linked immunosorbent assay.

PATIENTS AND METHODS

Patients

Between February, 2001 and September, 2002, 20 patients positive for HLA-A24 or -A2 with metastatic HRPC were entered into a Phase I study in which patients were treated by peptide-specific cytotoxic T lymphocyte (CTL) precursor oriented vaccination [15]. Thirteen patients were entered in the combined peptide vaccination and estramustine phosphate when the disease progressed after at least three peptide vaccinations in the Phase I study. The disease progression was defined by at least one of three criteria: two consecutive 25% increase from baseline PSA levels at least 2 weeks apart, a greater than 25% increase in bidimensionally measurable soft tissue metastases, or the appearance of new foci on radionuclide bone scans. Serum PSA levels were determined using Tandem-R (Hybritech, Inc., San Diego, CA) assays with a normal range between 0 and 4.0 ng/ml. Other eligibility included an Eastern Cooperative Oncology Group performance status of 0 or 1, age 79 years or less, white blood cell count greater than 3,000/mm^3, hemoglobin greater than 10 g/dl, platelets greater than 100,000/mm^3, bilirubin equal to or less than the institutional limit of normal, and creatinine less than 1.4 mg/dl. Negative serologic tests for hepatitis B and C were required. All patients had been untreated for at least 4 weeks before the study, and had an Eastern Cooperative Oncology Group performance status of 0–1. Patients with evidence of serious illness, an active secondary malignancy during 5 years before entry, immunosuppression by means of white blood cell count of lesser than 3,000, or autoimmune diseases were excluded from the study. All patients gave informed consent in accordance with institutional guidelines. This study was approved by the Kurume University School of Medicine ethics committee.

Patient-Oriented Peptide Vaccination

Our approach in the immunotherapy for HRPC patients is a pre-vaccination measurement of peptide-specific CTL precursors in the circulation of cancer patients reactive to 30 kinds of vaccine candidates (14 peptides for HLA-A24 positive patients, and 16 peptides for HLA-A2 positive patients) with the ability to induce CTLs, followed by administration of only reactive peptides (CTL precursor-oriented peptide vaccine) as reported previously [13–15]. The peptides used in the present study are listed in Table III. These peptides were prepared under conditions of Good Manufacturing Practice by a Multiple Peptide System

(San Diego, CA). All of these peptides have the ability to induce HLA-A24 or -A2-restricted and tumor-specific CTL activity in peripheral blood mononuclear cells (PBMCs) of the cancer patients [17–23]. Before the first vaccination and 7 days after every 6th vaccination, 30 ml of peripheral blood was obtained, 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 [24]. Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of each peptide in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V® medium (Invitrogen Corp., Carlsbad, CA), 10% FCS, 100 U/ml of interleukin-2 (IL-2), and 0.1 mM MEM nonessential amino acid solution (Invitrogen Corp.). Half of the medium was removed and replaced with the new medium containing a corresponding peptide (20 μM) every 3 days for up 12 days. On the 12th day of the culture, 24 hr after the last stimulation, these cells were harvested, washed three times, and then tested for their ability to produce IFN-γ in response to C1R-A2402 or C1R-A2 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLLGI) as a negative control in HLA-A24 or -A2 PBMCs, respectively. The target cells (C1R-A2402 or C1R-A2, 1×10^4/well) were pulsed with each peptide (10 μM) for 2 hr, and then effector cells (1×10^5/well) were added to each well with the final volume of 200 μl. After incubation for 18 hr, the supernatants (100 μl) were collected, and the amounts of IFN-γ were measured using an enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). All experiments were performed in the four different wells with duplicate assays. Pre-vaccination PBMCs were provided for screening of the CTL precursors reactive to 30 peptides (14 peptides for HLA-A24 positive patients, and 16 peptides for HLA-A2 positive patients) for different wells with duplicate assays in each well, and the results of each well were classified into four groups in accordance with the *P*-values (by two-tailed Student's *t*-test) and the amounts of IFN-γ (a mean value response to a corresponding peptide minus that to an HIV peptide) as follows: armed response (Ar): $P \leq 0.1$ and $500 \leq$ net; A level of response (A): $P \leq 0.05$ and $50 \leq$ net; B: $P \leq 0.05$ and $25 \leq$ net < 50; C: $0.05 < P \leq 0.1$ and $50 \leq$ net. Peptides were chosen based upon evaluation of all four wells by the order shown above.

CombinationTherapy

The peptide vaccination schedule was as follows. For the skin test, 10 μg of each selected peptide for up to four peptides were independently injected intradermally with a tuberculin syringe with a 27-gauge needle. Immediate- and delayed-type hypersensitivity (DHT) reactions were determined at 20 min and 24 hr after the skin test, respectively. A positive skin-test reaction was defined as >30-mm diameter erythema and induration, when saline was a negative control for assessment of the hypersensitivity. If immediate-type hypersensitivity was negative, the peptide was injected. Before the combination therapy, 3 mg/ml of each of the peptides was injected subcutaneously in the lateral thigh of each patient a total of six times at 2-week intervals. During the combination therapy, 1 mg/ml of each of the peptides was injected at 4- to 6-week intervals.

Estramustine phosphate was initially administered orally as 140-mg capsules, two capsules twice daily for a total daily dose of 560 mg for the first two cases, but severe immunosuppression by means of no IFN-γ production (<10 ng/ml) in response to the vaccinated peptides measurement of IFN-γ productions to in the PBMCs during the administration of estramustine phosphate at a dose of 560 mg per day. A part of the results are shown in a Case 2 of the Figure 2. To avoid the severe immnosuppression, estramustine phosphate was reduced to 280 mg/day for the remaining 11 cases.

Immunological Monitoring

For evaluation of immune responses during the combination therapy, peptide-specific CTL precursors in PBMCs and serum levels of peptide-specific antibodies were measured in every 6th vaccination. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method [24], while an ELISA was used to detect the serum IgG levels specific for the peptides administrated, as reported previously [13–15]. In addition, a new monitoring method was conducted to carefully measure the estramustine-induced immunosuppression. Namely, PBMCs were harvested every 2 weeks and were cultured (10^4 cells/well) for 2 days in triplicate assays with 10 μM of phytohemagglutinin (PHA), 10 ng/ml of Epstein-Bar virus (EBV)-derived peptide with HLA-A24 or -A2 binding motif, and 10 ng/ml of two different peptides under vaccination. After 2 days in culture, the amounts of IFN-γ in cell free supernatants were measured in triplicate assays, and viable cell numbers were also counted. To avoid biases in each assay, all the PBMCs were once cryopreserved and the four different PBMCs (two from healthy donors, one from patients PBMCs harvested 2 weeks before the latest vaccination, and one from the latest vaccination) were thawed at the same time in the morning of the experiment. The PBMCs from HD were from the same donors throughout the series of immuno monitoring.

Responses (IFN-γ production) to PHA, EBV-peptide, and the vaccinated peptides were considered to be mediated by resting T-cells, memory T-cells, and a combination of memory and activated T-cells based on the criteria mentioned by Kaech et al. [25], respectively.

Clinical Monitoring

Patients were observed until disease death or intolerance, or consent was withdrawn. Clinical and laboratory assessments were performed at each visit, and patients were questioned about adverse events, their severity, and frequency. The severity of the adverse events was scored according to the National Cancer Institute (NCI) Toxicity Criteria. Serum PSA and bone turnover marker (pyridinoline cross-linked carboxyterminal telopeptide of Type I collagen: ICTP) levels were measured every 4 weeks during the treatment. The serum levels of ICTP were measured using a two-antibody radioimmunoassay (RIA) using the Telopeptide ICTP RIA kit (Orion Diagnostica, Espoo, Finland, provided by Chugai, Tokyo, Japan). The normal range of serum ICTP was 1.8–5.0 ng/ml [26]. Bone scans and computed tomography (CT) scans of the abdomen were performed every 3 months during this study. The metastatic findings on bone scans were assessed by the extent of the disease using the percentage of positive area on the bone scan (%PABS) [27]. Clinical response was determined by both changes in PSA levels and by imaging studies in patients with measurable disease. PSA response was defined as two consecutive measurements at least 4 weeks apart that showed a 50% or greater decrease from baseline PSA levels (partial response (PR)) or normalization of the PSA level (complete response (CR)). Time of PSA progression was registered at the time of the first of two consecutive PSA levels 25% above the baseline. Standard definitions were used for response and progression of measurable and evaluable disease. For patients with bidimensionally measurable disease, a CR was defined as disappearance of all target lesions for at least 4 weeks; a PR was defined as a $\geq$50% decline in bidimensionally measurable disease, and a minor response was defined as a reduction between 25% and 50%. For a response of bone metastasis, a CR was the defined as disappearance of all positive areas on bone scans. A PR was defined as a 50% or greater decrease in the %PABS, and progression (PD) was defined as an increased number of positive sites, increased intensity of the existing lesions, or the two findings observed concurrently.

Statistical Methods

Progression-free survival was defined as the time from the beginning of the combination therapy to the time of progression for patients whose disease progressed, to the time of death for patients who died progression free, or to the time of the last contact who remained alive and progression free. Cause-specific survival was defined as the time from the beginning of the combination therapy to disease caused death. The Kaplan–Meier method was used to estimate progression-free and cause-specific survival.

RESULTS

Patient Characteristics

A total of 13 patients positive for HLA-A24 or -A2 with metastatic HRPC who had failed to respond to the prior-peptide vaccination were enrolled in this study. All 13 patients were included in toxicity assessments. Two patients were withdrawn from the immunological and clinical evaluation because of incompletion of the intended course of therapy (6th vaccination) and there was no sample for the immunological analysis. Therefore, 11 patients were assessable for immunological and clinical evaluation. Baseline characteristics of 11 patients treated by the combination therapy are summarized in Table I. At the time of enrollment in the study, the median age was 71 years (range, 57–75 years), and median Eastern Cooperative Oncology Group (EOCG) performance status was 0. Median serum PSA and ICTP levels were 330 ng/ml (range, 27–1,072 ng/ml) and 6.2 ng/ml (range, 3.0–15.3 ng/ml),

TABLE I. Patient Characteristics

No. of patients	11
Age (year)	
Median	71
Range	57–75
EOCG performance status (n)	
0	8
1	3
HLA typing (n)	
A24	4
A2	7
Serum PSA level (ng/ml)	
Median	330
Range	27–1,072
Serum ICTP level (ng/ml)	
Median	6.2
Range	3.0–15.3
Site of metastases (n)	
Bone only	9
Bone and nodal/organ	1
Nodal/organ	1
Prior use of estramustine phosphate (n)	
Yes	8
No	3

respectively. Of the 11 patients with metastatic HRPC, 9 had only bone involvement, 1 had bone plus nodal/organ involvement, and the remaining 1 had nodal/organ disease without bone involvement. Median %PABS in patients with bone metastases was 6.0 (range, 1.5–8.4). Prior treatments included hormonal therapy (11 patients), radiation therapy to bone metastases (2 patients), and chemotherapy with estramustine phosphate (eight patients of whom five patients were treated with estramustine alone and three patients were treated with estramustine plus a combination of etoposide). All 11 patients also received more than 3 (median 6, range 3–23 times) peptide vaccinations before the start of the combination therapy. The median duration of the combination therapy was 13 months (range, 6–21 months).

Toxicity

The toxicities reported among the 13 treated patients are summarized in Table II. One case (Case 4) of Grade 3 arrhythmia and one case (Case 7) of Grade 3 cerebral infaction were observed. The arrhythmia disappeared by discontinuation of the estramustine. The other patient (Case 7), who was hospitalized with Grade 3 cerebral infarction after the 14-month-combination therapy, was successfully treated with anticoagulants, and was continuing the combination therapy without the other major toxicities. The most common toxicities were dermatologic reactions at the injection site of the vaccination in all cases. All 13 dermatologic reactions were scored as Grade 1 or 2 using National Cancer Institute common toxicity criteria. Seven patients complained of bone pain, four patients developed Grade 2 hematuria and three patients complained of fatigue.

TABLE II. Adverse Events of Combination of Patient-Oriented Vaccination and Oral Estramustine

	Grade[a]				
Toxicity	1	2	3	4	Total
Dermatologic	10	3			13
Bone pain	3	4			7
Hematuria		4			4
Fatigue	1	2			3
Nausea	2				2
Diarrhea	2				2
Chest pain	2				2
Edema	2				2
Arrhythmia			1		1
Cerebral infarction			1		1
Fever	1				1

[a]Toxicities based on the National Cancer Institute common toxicity scale. Some patients had more than one toxic reaction.

There was no treatment related to hematologic, hepatic, or renal toxicity.

Immunological Response During the CombinationTherapy

During the combination therapy, peptide-specific CTL precursors and peptide-specific antibodies were measured at 6-week intervals in all 11 patients. Vaccinated peptides and immune responses are summarized in Table II. All 11 patients monitored for immune response during the combination therapy had either enhanced cellular or humoral responses. Augmentation of peptide-specific CTL precursors was observed in 6 of 11 patients (Cases 2, 5, 7, 8, 10 and 12), while induction of peptide-specific IgG was observed in 10 of 11 patients (Cases 3, 4, 5, 7, 8, 10, 11, 12, and 13). Figure 1 demonstrates serial changes of both IFN-γ productions and IgG levels specific for the peptides administrated in each patient.

The estramustine-induced immunosuppression was also analyzed in 10 of 11 patients by measurement of IFN-γ productions to PHA, EBV-peptide, and the vaccinated peptides. Immunomonitoring was not carried out for Case 13 because the available PBMCs were too few for use in the assay. Responses (IFN-γ productions) to PHA, EBV-peptide, and the vaccinated peptides were suggested to be mediated by resting T-cells, memory T-cells, and a combination of memory and activated T-cells, respectively. Results of monitoring in each case are shown in Figure 2. Cases 2 and 3 were initially treated by the combination with a full dose (560 mg/day) of estramustine phosphate, but the immunological monitoring revealed severe immune suppression. These immune suppressions were recovered by discontinuing administration of estramustine phosphate. There was no significant immune suppression in any of eight cases tested when the peptide and a half dose of estramustine phosphate were administrated (Fig. 2).

Clinical Response

Clinical responses to the combination of peptide vaccination and oral estramustine phosphate are presented in Table IV. Ten of 11 (91%) patients showed a serum PSA level decrease from the baseline after the treatment including eight patients (73%) who showed a serum PSA level decrease of ≥50%. Serial changes of PSA levels in each case during the combination therapy are shown in Figure 3. PSA responses were noted in all eight patients who had failed prior chemotherapy with estramustine phosphate. One of two patients with measurable disease showed a 44% decrease of lymph node metastasis on the CT (Fig. 4). This patient is still alive with a PSA decline of ≥50% (Case 12). Ten

TABLE III. Immune Response During the CombinationTherapy

Case (patients no.)	HLA type	Peptide	Sequence	Cellular response to peptide[a] during the combination therapy				Antipeptide IgG during the combination therapy				DTH induction
				Pre	6th	12th	18th	Pre	6th	12th	18th	
2 (010)	A-24	$lck_{488-497}$	DYLRSVLEDF	ArAB	–	AC	–	–	–	–	–	–
		$SART2_{93-101}$	DYSARWNEI	C	–	–	–	–	–	–	–	–
		$SART2_{161-169}$	AYDFLYNYL	A	–	AAC	ArArAA	–	–	–	–	+ (20)
		$ART1_{170-179}$	EYCLKFTKL	A	C	AAA	A	++	++	++	++	+ (17)
		$lck_{208-216}$	HYTNASDGL	–	–	–	–	–	–	–	–	+ (16)
		$SART3_{315-323}$	AYIDFEMKI	–	–	Ar	ArArArB	–	–	–	–	–
3 (014)	A-24	$SART2_{93-101}$	DYSARWNEI	–	–	n.a.	n.a.	–	–	n.a.	n.a.	–
		$SART3_{109-118}$	VYDYNCHVDL	ArAA	ArA	n.a.	n.a.	+	++	n.a.	n.a.	–
		$SART3_{315-323}$	AYIDFEMKI	AC	A	n.a.	n.a.	+	+	n.a.	n.a.	–
		CyB_{91-99}	DFMIQGGDF	–	C	n.a.	n.a.	–	–	n.a.	n.a.	–
		$lck_{208-216}$	HYTNASDGL	A	–	n.a.	n.a.	–	–	n.a.	n.a.	–
		$lck_{488-497}$	DYLRSVLEDF	C	–	n.a.	n.a.	–	–	n.a.	n.a.	–
4 (016)	A-24	$ART1_{170-179}$	EYCLKFTKL	A	A	–	–	++	++	++	++	+ (21)
		$lck_{488-497}$	DYLRSVLEDF	B	–	–	–	–	–	–	+	+ (23)
		$SART3_{109-118}$	VYDYNCHVDL	–	–	B	–	–	+	++	++	+ (21)
		$SART2_{161-169}$	AYDFLYNYL	–	Ar	B	–	–	–	–	–	–
5 (019)	A-24	$SART\ 2_{161-169}$	AYDFLYNYL	A	–	–	ArA	–	–	–	–	+ (8)
		$SART2_{899-907}$	SYTRLFLIL	A	–	–	A	–	–	–	–	+ (7)
		$SART3_{109-118}$	VYDYNCHVDL	ArArArA	ArArArA	ArArArA	ArArAA	–	–	–	+	+ (8)
		$lck_{208-216}$	HYTNASDGL	A	AA	–	–	–	–	–	+	+ (5)
		$lck_{486-497}$	DYLRSVLEDF	–	ABB	–	–	–	+	+	+	+ (6)
7 (104)	A-2	$ppMAPkkk_{432-440}$	DLLSHAFFAI	–	C	–	n.a.	+	++	++	n.a.	+ (32)
		$lck_{246-254}$	KLVERLGAA	–	–	B	n.a.	++	++	++	n.a.	+ (31)
		$lck_{422-430}$	DVWSFGILL	A	AB	–	n.a.	–	+	+	n.a.	+ (31)
		$UBE2V_{43-51}$	RLQEWXSVIL	–	ACC	ArArArA	n.a.	++	++	++	n.a.	+ (11)
		$HNRPL_{140-148}$	ALVEFEDVL	AAC	AC	ArArArA	n.a.	–	++	+	n.a.	–
		$HNRPL_{501-510}$	NVLJFFNAPL	–	AAA	ArArAc	n.a.	–	++	++	n.a.	–
8 (108)	A-2	$lck_{422-430}$	DVWSFGILL	Ar	–	–	C	–	–	–	–	+ (3)
		$PpMAPkkk_{294-302}$	GLLFLHTRTI	CC	–	–	–	–	+	+	+	+ (2)
		$PpMAPkkk_{432-440}$	DLLSHAFFAI	ArA	A	–	–	–	–	+	+	+ (3)
		$HNRPL_{501-510}$	NVLJFFNAPL	–	AB	E	ArA	–	–	–	+	+ (17)
		$CypB_{172-179}$	VLEGMEVV	A	–	–	B	–	+	+	+	–
		$EIF4EBP1_{51-59}$	RIIYDRKFL	–	–	A	B	–	–	–	+	–

(*Continued*)

TABLE III. (*Continued*)

Case (patients no.)	HLA type	Peptide	Sequence	Cellular response to peptide[a] during the combination therapy				Antipeptide IgG during the combination therapy				DTH induction
				Pre	6th	12th	18th	Pre	6th	12th	18th	
9 (111)	A-2	$lck_{422-430}$	DVWSFGILL	C	–	n.a.	n.a.	–	–	n.a.	n.a.	+ (5)
		$PpMAPkkk_{294-302}$	GLLFLHTRTI	A	–	n.a.	n.a.	–	–	n.a.	n.a.	+ (5)
		$PpMAPkkk_{432-440}$	DLLSHAFFAI	A	–	n.a.	n.a.	–	–	n.a.	n.a.	+ (6)
		$WHSC2_{103-111}$	ASLDSDPWV	CC	–	n.a.	n.a.	–	–	n.a.	n.a.	–
		$HNRPL_{501-510}$	NVLJFFNAPL	A	–	n.a.	n.a.	–	–	n.a.	n.a.	–
		$CypB_{129-138}$	KLKHYGPGWV	–	–	n.a.	n.a.	–	–	n.a.	n.a.	–
10 (112)	A-2	$SART3_{309-317}$	RLAEYQAYI	A	Ar	Ar	ArAC	–	–	–	+	–
		$CypB_{172-179}$	VLEGMEVV	A	–	–	A	–	+	+	+	–
		$lck_{246-254}$	KLVERLGAA	–	–	–	ArAAA	–	–	–	–	–
		$lck_{422-430}$	DVWSFGILL	–	C	–	ArAAA	–	+	+	–	–
		$PpMAPkkk_{294-302}$	GLLFLHTRTI	–	C	–	ArArAA	–	–	–	–	–
		$UBE2V_{85-93}$	LIADFLSGLI	–	–	A	AC	–	–	+	+	–
11 (113)	A-2	$CypB_{129-138}$	KLKHYGPGWV	A	–	–	n.a.	–	–	–	n.a.	+ (6)
		$lck_{246-254}$	KLVERLGAA	A	–	–	n.a.	–	++	++	n.a.	–
		$lck_{422-430}$	DVWSFGILL	A	A	C	n.a.	–	–	–	n.a.	–
		$PpMAPkkk_{294-302}$	GLLFLHTRTI	–	–	C	n.a.	+	++	++	n.a.	+ (6)
		$UBE2V_{43-51}$	RLQEWXSVIL	AAA	–	–	n.a.	–	++	++	n.a.	+ (10)
12 (115)	A-2	$CypB_{129-138}$	KLKHYGPGWV	–	A	–	n.a.	–	–	+	n.a.	–
		$lck_{246-254}$	KLVERLGAA	BB	A	B	n.a.	++	++	++	n.a.	–
		$WHSC2_{141-}$	ILGELREKV	AB	–	–	n.a.	–	–	–	n.a.	–
		$UBE2V_{43-51}$	RLQEWXSVIL	AAAB	–	A	n.a.	–	++	++	n.a.	+ (19)
		$HNRPL_{501-510}$	NVLJFFNAPL	ArAr	ArArArAr	ArArAr	n.a.	+	+	++	n.a.	+ (19)
		$EIF4EBP1_{51-59}$	RIIYDRKFL	–	–	A	n.a.	+	++	++	n.a.	–
13 (116)	A-2	$CypB_{172-179}$	VLEGMEVV	–	–	A	n.a.	+	+	+	n.a.	–
		$lck_{246-254}$	KLVERLGAA	ArC	AC	ArAA	n.a.	++	++	++	n.a.	–
		$lck_{422-430}$	DVWSFGILL	Ar	AAA	–	n.a.			+	–	–
		$UBE2V_{43-51}$	RLQEWXSVIL	ArA	AA	C	n.a.	+	+	++	n.a.	–
		$ppMAPkkk_{432-440}$	DLLSHAFFAI	–	A	–	n.a.	–	+	+	n.a.	+ (17)

–, absent; +, present, low titer; ++, present, high titer.
n.a., not available.
AAAA, all four wells of quadruplicate assay were positive.
AAA, three wells of quadruplicate assay were positive.
AA, two wells of quadruplicate assay were positive.
A, one well of quadruplicate assay were positive.
[a]The CTL precusor assay was performed and each well was evaluated by the following criteria, and up to four peptides were administered; Ar: $P \leq 0.1$ (Student's *t*-test) and $500 \leq$ net (specific IFN-γ production (pg/ml) was calculated by subtracting the response to HIV-derived irrelevent peptide); A: $P \leq 0.05$ and $50 \leq$ net; B: $P \leq 0.05$ and $25 \leq$ net < 50; C: $0.05 < P < 0.1$ and $50 \leq$ net.

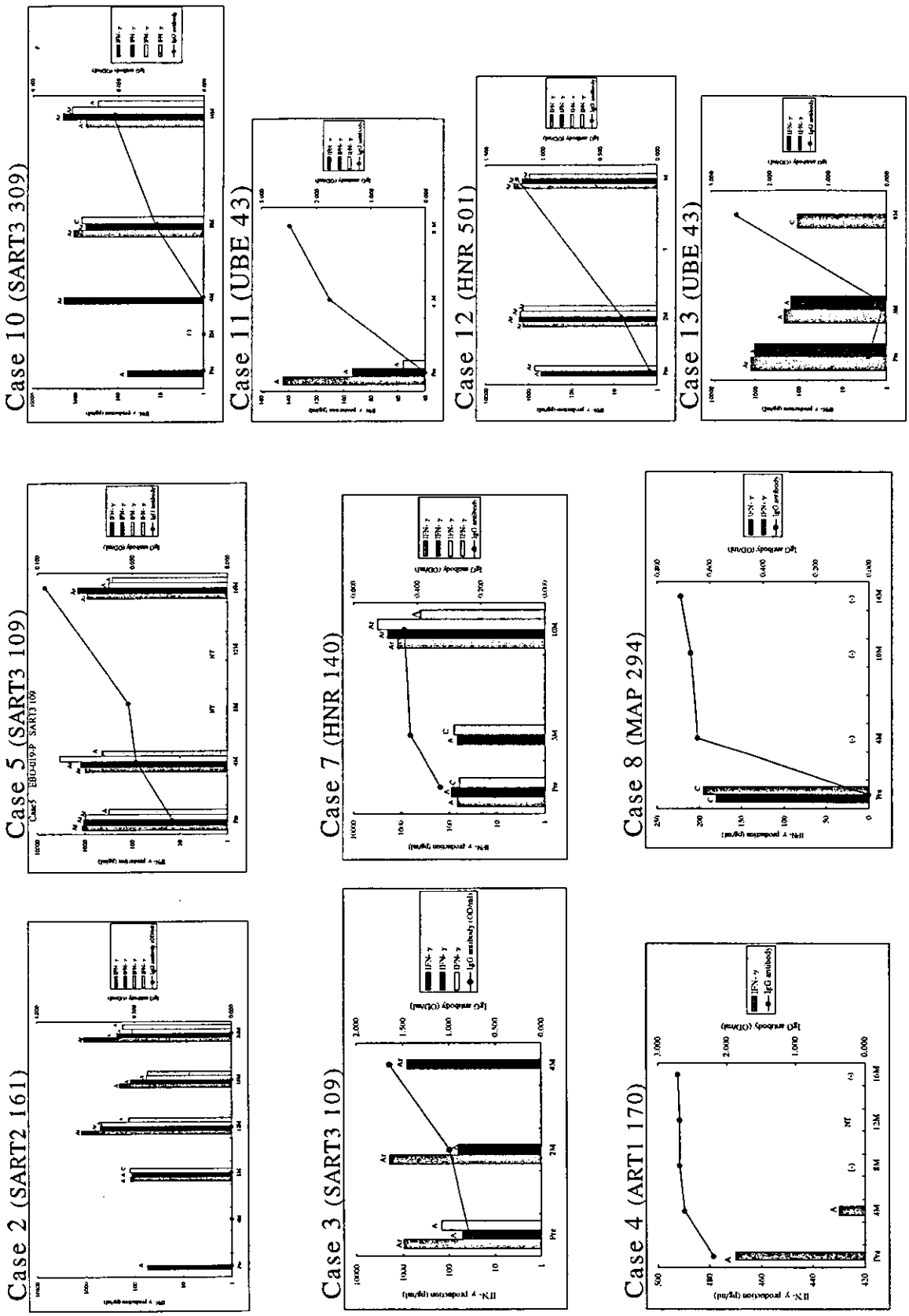

Fig. 1. Serial changes of IFN-γ productions and IgG levels specific for the peptides administered in each case. Augmentation of peptide-specific CTL precursors was observed in Cases 2, 5, 7, 8, 10, and 12, while induction of peptide-specific IgG was observed in Cases 3, 4, 5, 7, 8, 10, 11, 12, and 13.

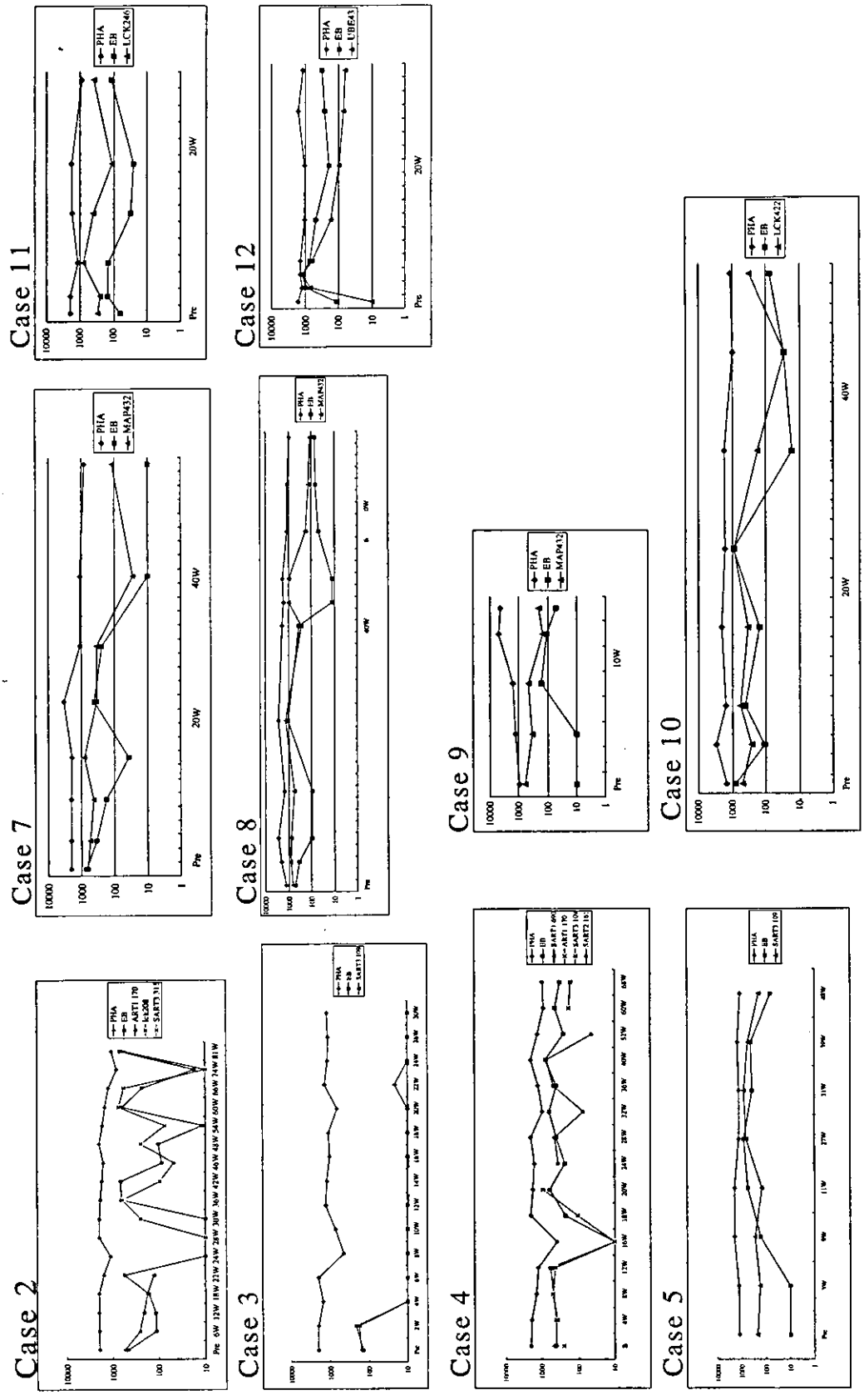

Fig. 2. Monitoring of the treatment-induced immune suppression. Cases 2 and 3 revealed severe immune suppression and their immune suppressions were recovered by discontinuing administration of full dose (560 mg/day) estramustine phosphate. There was no immune suppression in any of eight cases when the peptide and low-dose (280 mg/day) estramustine phosphate was administered.

TABLE IV. Best Response of PSA, ICTP, Bone Scan, and Measurable Disease During Combination Therapy

Type of response (no. of evaluable patients)	No. of patients (%)
PSA response (n = 11)	
≥50% decline	8 (73)
<50% decline	2 (18)
Total	10 (91)
ICTP response (n = 10)	
≥50% decline	1 (10)
<50% decline	8 (80)
Total	9 (90)
Bone scan response (n = 10)	
≥50% decline of %PABS	0
<50% decline of %PABS	0
Total	0
Measurable disease response (n = 2)	
≥50% decline	0
<50% decline	1 (50)
Total	1 (50)

patients had bone metastases. There was no improvement of bone metastases, although 1 of 10 patients with bone metastases showed a serum ICTP level decrease of ≥50%.

At present, three patients have died and all deaths were attributed to prostate cancer or metastases. The median follow-up for all patients was 14 months, ranging from 8 to 24 months. The median survival rate has not been calculated. At 12 months, 64% of patients were still alive.

DISCUSSION

The results of this study suggest a benefit of combination of peptide vaccination and a low dose (280 mg/day) of estramustine phosphate in patients with metastatic HRPC who had received the prior vaccination. Combination of immunotherapy and cytotoxic drugs is not a new concept [28], but there have been major concerns about a negative interaction which might take place due to the myelosuppressive properties of many cytotoxic drugs. Cytotoxic drugs also preferentially kill cells in division, a hallmark of an activated immune system, and therefore could inhibit immune responses. However, myelosuppression has rarely been reported as a toxicity of estramustine phosphate in patients treated for HRPC [16]. In a Phase III study of estramustine phosphate combined with vinblastine versus vinblastine alone, the rate of neutropenia was lower in the combination arm versus the monotherapy arm (Grades 2, 3, 4: 7%, 1%, and 1% vs. 27%, 18%, and 9%, respectively) [3]. In addition, the present study demonstrated that cellular and humoral responses were well maintained in all patients with metastatic HRPC during the combination of peptide vaccination and a low-dose estramustine phosphate. The present results showed that augmentation of peptide-specific CTL precursors was observed in 6 of 11 patients and induction of peptide-specific IgG was observed in 10 of 11 patients. There was no significant immune suppression in any of 11 patients when the peptide and low dose of estramustine phosphate were administered. Further studies with a relatively large number of patients are recommended to confirm the results from this small-scale study.

Defining the expression of tumor antigens on prostate cancers of different stages is the crucial first step in selecting targets for specific immunotherapy [29–31]. The present approach in immunotherapy for HRPC patients used a new strategy of a pre-vaccination measurement of peptide-specific CTL precursors in the circulation of cancer patients, followed by administration of up to four peptides that had been reactive for pre-vaccination measurement among 30 vaccine candidates (patient-oriented vaccination). Previous results from a Phase I study demonstrated that patient-oriented vaccination is feasible, safe, and immunologically active but the clinical response has been largely limited [15]. It has been known for some time that the malignant transformation of cells is associated with altered HLA Class I expression and/or function, and that these abnormalities can provide tumor cells with avenues of escape from immune recognition. In contrast to the normal HLA Class I expression of the benign tissue, complete loss of HLA Class I expression was reported in 34% of primary prostate cancer cells and 80% of prostate cancer cells of lymph node metastases [32]. Therefore, HLA Class I antigen down-regulation in prostate cancer may have a negative impact on the outcome of T-cell-based immunotherapy because they provide malignant cells with a mechanism by which to escape T-cell recognition. It is suggested that additive antitumor effects could be achieved by the combination of T-cell-based immunotherapy and cytotxic agents with minimum immunosuppression. In the present study, PSA responses were observed in patients who experienced disease progression prior to estramustine phosphate or peptide vaccination, supporting the hypothesis that this combination works by additive antitumor effects. However, the exact mechanism of this interaction is unclear. Further studies on this mechanism are needed.

The overall response rate (73%) defined as a serum PSA level decrease of ≥50% is significantly higher than those seen in previously reported Phase I/II studies of immunotherapy such as the combination of interferon-α and interleukin-2 therapy (31%) [33] or the infusion of

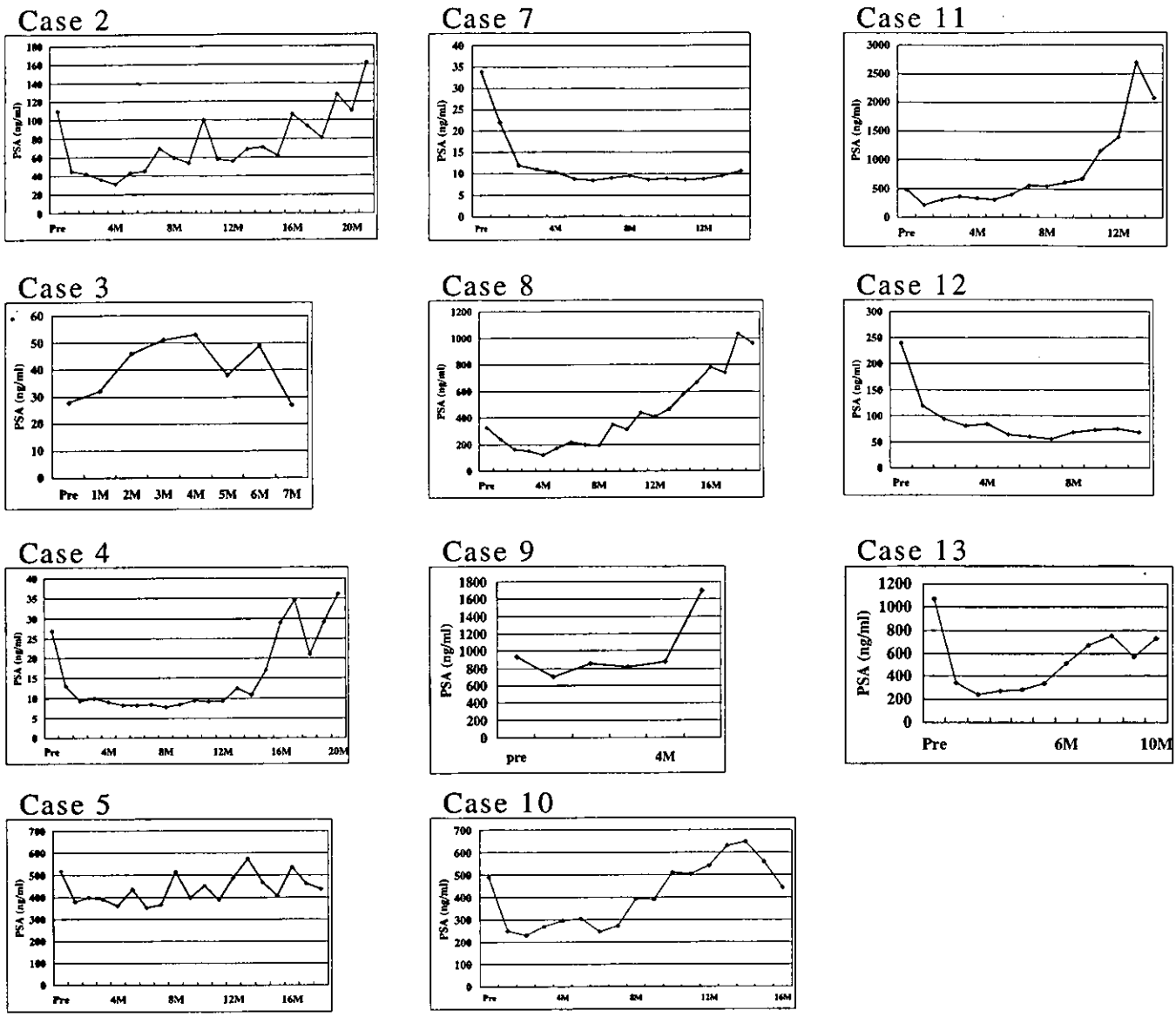

Fig. 3. Serial changes of PSA levels in 11 patients during the study. Ten of 11 (94%) patients showed serum PSA level decrease from baseline during the combination therapy.

dendric cells primed with peptides of prostate-specific membrane antigen (27%) [11,12]. Moreover, it is also comparable with the response rate in recently reported chemotherapy trials with combinations such as estramustine and paclitaxel (53%) [6], estramustine and docetaxel (62%) [7], and the three-drug combination of estramustine, peclitaxel, and carboplatin (67%) [8]. In terms of measurable disease, the overall response rate appears somewhat lower than that reported with those chemotherapy regimens because few patients in the present study had measurable soft tissue disease. Although, the majority of patients showed decreased bone turnover marker (ICTP) that were proposed as a modality for monitoring bone metastasis in patients with prostate cancer with bone metastasis [34], there was no improvement of bone metastasis on bone scans. Possible explanations for this discrepancy are that bone scans are an insensitive tool or that the duration of the treatment was too short to affect the disease in bone where it can be more resistant to therapy.

The toxicity of the combination regimen reported here was less and this treatment was considered acceptable in the treatment of the vast majority of metastatic HRPC. The most common toxicities were dermatologic reactions at the injection site of the vaccination. Importantly, there was no hematologic toxicity nor neuropathy reported in estramustine-based or taxane-based chemotherapy regimens, and

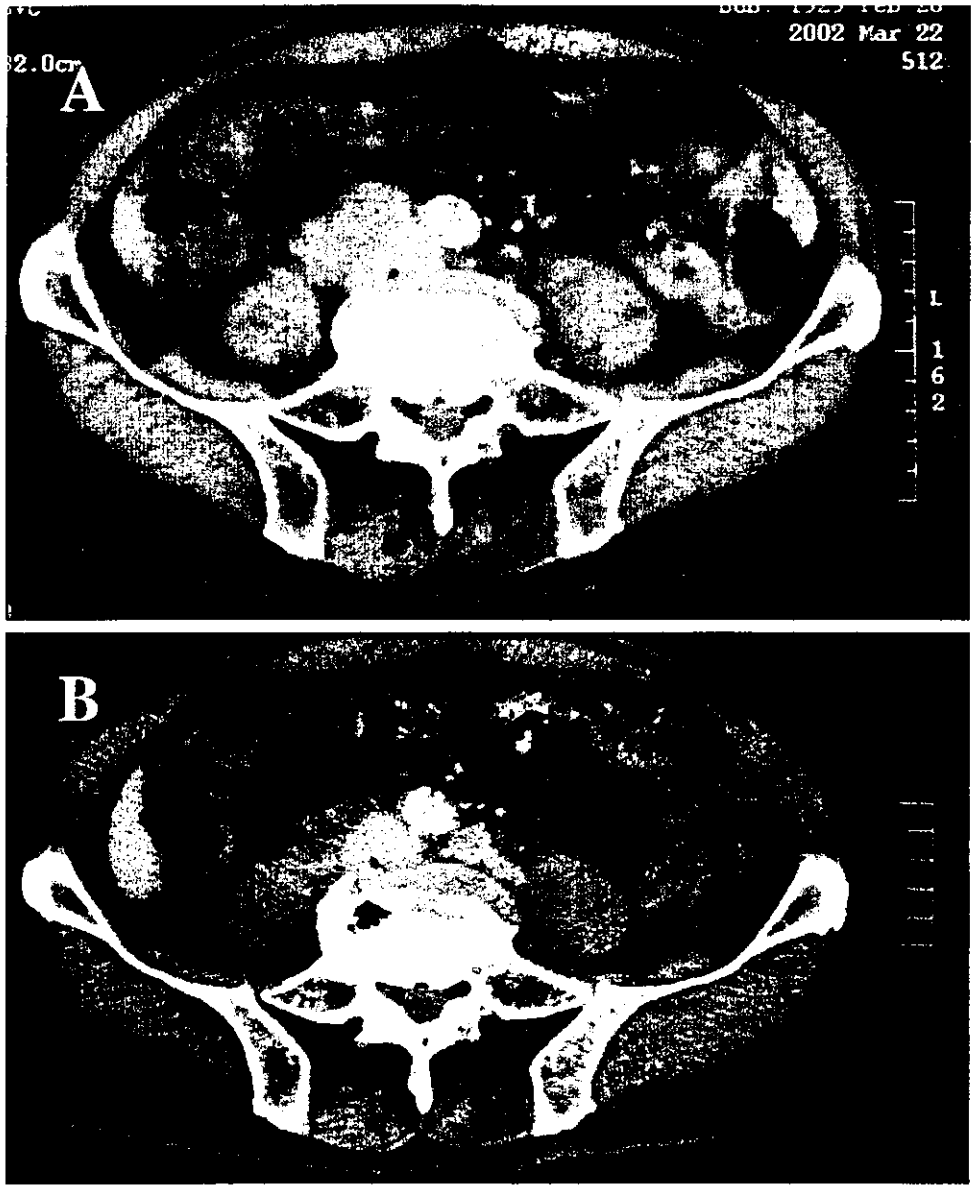

Fig. 4. CT images of Case 12. **A**: Para-aortic lymph node metastasis (arrow) was detected by a CT scan at the beginning of combination therapy. **B**: A repeat CT scan after 8 months with the combination therapy showed a 44% decrease in the size of lymph node metastasis (arrow).

they were dose-limiting toxicities [4–8]. The common toxicities of estramustine treatment include nausea, vomiting, peripheral edema, and vascular events [16].

The combination of patient-oriented vaccination and low-dose estramustine phosphate was associated with a serum PSA level decrease of ≥50% in 73% of the metastatic HRPC who had received the prior peptide-vaccination. Based on these preliminary findings, larger Phase II studies of this regimen are warranted.

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The Prostate 60:205–213 (2004)

A Prostate Stem Cell Antigen-Derived Peptide Immunogenic in HLA-A24$^-$ Prostate Cancer Patients

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BACKGROUND. We attempted to identify prostate stem cell antigen (PSCA)-derived peptides immunogenic in HLA-A24$^+$ prostate cancer patients.
METHODS. Peripheral blood mononuclear cells (PBMCs) were stimulated in vitro with each of three different PSCA-derived peptides, which were prepared based on the HLA-A24 binding motif, and their peptide-specific and HLA-A24-restricted anti-tumor responses were examined. Plasma levels of immunoglobulin G (IgG) against PSCA peptides were measured by enzyme-linked immunosorbent assay (ELISA).
RESULTS. Among three PSCA peptides, the PSCA 76–84 peptide most effectively induced peptide-specific cytotoxic T lymphocytes (CTLs) from PBMCs of HLA-A24$^+$ prostate cancer patients. Cytotoxicity was dependent on peptide-specific and CD8$^+$ T cells. The PSCA 76–84 peptide-stimulated PBMCs showed a significant level of cytotoxicity against prostate cancer cells in an HLA-A24-restricted manner. IgG reactive to the PSCA 76–84 peptide was detected in half of patients.
CONCLUSIONS. The PSCA 76–84 peptide should be considered for use in clinical trials of immunotherapy for HLA-A24$^+$ patients. *Prostate 60: 205–213, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; PSCA; cytotoxic T lymphocytes; HLA-A24; peptide; antibody**

INTRODUCTION

Prostate cancer is one of the most common cancers in older men [1]. Although hormone therapy can temporarily palliate prostate cancer, progression to hormone-refractory prostate cancer (HRPC) is observed in most cases [2]. Therefore, novel therapeutic modalities for the treatment of HRPC are required. One such therapy could be peptide-based immunotherapy, as recent advances in tumor immunology have enabled us to identify many genes encoding tumor antigens and their peptides that are recognized by cytotoxic T lymphocytes (CTLs) [3,4]. Regarding prostate-related antigens, several antigenic peptides derived from either prostate-specific antigen (PSA) [5–7], prostate-specific membrane antigen (PSMA) [8,9], or prostatic acid phosphatase (PAP) [10,11] have been identified. Some of these antigen-derived peptides have been used in the treatment of prostate cancer patients, but the clinical responses observed thus far have been unsatisfactory [12–14].

Prostate stem cell antigen (PSCA) is a recently identified antigen expressed on the cell surface of

Abbreviations: CTLs, cytotoxic T lymphocytes; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; HRPC, hormone-refractory prostate cancer; IFN, interferon; IL, interleukin; Ig, immunoglobulin; mAb, monoclonal antibody; OD, optical density; PAP, prostatic acid phosphatase; PBMCs, peripheral blood mononuclear cells; PSA, prostate-specific antigen; PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen.

Grant sponsor: Ministry of Health, Labor, and Welfare of Japan (for Cancer Research (15–17)); Grant sponsor: Ministry of Education, Science, Sports, and Culture of Japan.

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Received 7 October 2003; Accepted 2 December 2003
DOI 10.1002/pros.20038
Published online 2 February 2004 in Wiley InterScience (www.interscience.wiley.com).

prostate cancer cells [15]. This antigen is a glycosylphoshatidylinositol-anchored protein, and is overexpressed by both androgen-dependent and androgen-independent prostate cancers [15]. In addition, the expression of PSCA in prostate cancer is higher in metastases than in primary tumors [16]. These findings indicate that this antigen could be a good target molecule in specific immunotherapy for patients with HRPC and/or bone metastases. In this study, we tried to identify new PSCA-derived peptides that have the potential to elicit immune responses in HLA-A24$^+$ prostate cancer patients.

MATERIALS AND METHODS

Patients

All HLA-A24$^+$ prostate cancer patients participating in this study provided their informed consent prior to enrollment. None of these participants was infected with human immunodeficiency virus (HIV). Twenty milliliters of peripheral blood was obtained, and peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll–Conray density gradient centrifugation. The expression of HLA-A24 molecules on PBMCs of cancer patients was determined by flow cytometry.

Cell Lines

C1R-A24 is an HLA-A*2402-expressing cell line. PC93 is an HLA-A24-negative prostate cancer cell line (HLA-A*6802$^+$) that was established by Dr. K. Ohishi (Department of Urology, Kyoto University, Japan). PC93-A24 is a subline that was stably transfected with the *HLA-A*2402* gene. The expression of HLA-A2402 molecules on PC93-A24 was previously reported [10]. Both Colo201 and Colo302 are HLA-A24$^-$ and HLA-A24$^+$ colon carcinoma cell lines, respectively. All cell lines were maintained in RPMI-1640 medium (Gibco-BRL, Gaithersberg, MD) supplemented with 10% FCS.

Flow Cytometric Analysis

To examine the expression of HLA-A24 molecules on tumor cell lines, cells were stained by anti-HLA-A24 monoclonal antibody (mAb), followed by FITC-conjugated goat anti-mouse immunoglobulin G (IgG). The results were analyzed using the program CELLQuest (Becton Dickinson, Mountain View, CA).

Peptides

Three PSCA-derived peptides, which are listed in Table I, were prepared based on the HLA-A*2402 binding motif [17]. All peptides were of >90% purity and were purchased from the Biologica Co. (Nagoya, Japan). Influenza (Flu) virus-derived (RFYIQMCYEL), Epstein–Barr virus (EBV)-derived (TYGPVFMCL), and HIV-derived (RYLRQQLLGI) peptides with the HLA-A24 binding motif were used as a control. All peptides were dissolved with dimethyl sulfoxide at a dose of 10 mg/ml.

Assay for Peptide-Specific CTLs in PBMCs

The assay for the detection of peptide-specific CTLs was performed according to a previously reported method with several modifications [18]. In brief, PBMCs (1×10^5 cells/well) were incubated with 10 μg/ml of each peptide in quadruplicate in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The culture medium consisted of 45% RPMI 1640, 45% AIM-V medium (Gibco-BRL), 10% FCS, 100 U/ml of interleukin-2 (IL-2), and 0.1 mM MEM nonessential amino acid solution (Gibco-BRL). Half of the culture medium was removed and replaced with new medium containing a corresponding peptide (20 μg/ml) every 3 days. On the 15th day of culture, the cultured cells were separated into 4-wells, with 2-wells being used for PSCA peptide-pulsed C1R-A24 cells (1×10^4 cells/well) and the other 2-wells being used for the HIV peptide-pulsed C1R-A24 cells. After an 18-hr incubation, the supernatant was collected, and the level of interferon (IFN)-γ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/ml).

In Vitro Culture for CTL Assay

PBMCs from patients (1×10^5 cells/well) were incubated with 10 μg/ml of each peptide in 12-wells in a U-bottom-type 96-well microculture plate (Nunc) at a volume of 200 μl of the culture medium containing 100 U/ml of IL-2. Half of the culture medium was removed and replaced with new medium containing a corresponding peptide (20 μg/ml) and IL-2 (100 U/ml) every 3 days. On the 15th day of culture, half of the cultured cells were harvested from each well, and the cultured cels from 4-wells were pooled and examined for the reactivity to a corresponding PSCA peptide based on their IFN-γ production. Then, those pooled cells, which produced a significant level of IFN-γ in response to the corresponding PSCA peptide, were further cultured with IL-2(100 U/ml) in order to obtain a sufficiently large number of cells to carry out a CTL assay.

Cytotoxicity Assay

Cultured cells were tested for their cytotoxicity against both PC93 and PC93-A24 by a 6-hr ^{51}Cr-release assay. Two thousand ^{51}Cr-labeled cells per well were

TABLE I. Reactivity of PSCA Peptide-Stimulated PBMCs From HLA-A24$^+$ Prostate Cancer Patients

	Peptides					
	Names	PSCA 27–36	PSCA 76–84	PSCA 82–91	EBV	Flu
Prostate cancer patients	Amino acid sequence	KAQVSNEDCL	DYYVGKKNI	KNITCCDTDL	TYGPVFMCL	RFYIQMCYEL
	Score[a]	12.0	50.0	12.0		
				IFN-γ production (pg/ml)[b]		
#1		102	142	0	102	0
#2		0	47	0	162	120
#3		0	140	0	0	162
#4		0	0	0	203	0
#5		0	27	0	27	281
#6		0	39	0	460	0
#7		0	168	0	168	0
#8		0	144	0	114	233
#9		0	181	0	197	0
#10		154	0	0	124	0
#11		0	187	0	174	0
	Total	2/11	6/11	0/11	9/11	4/11

PSCA, prostate stem cell antigen; PBMCs, peripheral blood mononuclear cells; IFN, interferon.
[a]Scores represent the estimated half-time of the PSCA peptides binding HLA-A24 molecules.
[b]PBMCs from HLA-A24$^+$ prostate cancer patients were stimulated in vitro with the indicated PSCA petides as described in the Materials and Methods.
On day 15, the cultured PBMCs were tested for their reactivity to CIR-A24 cells, which were pre-pulsed with a corresponding peptide in quadruplicate.
Value represent the means of IFN-γ production by the effector cells in response to CIR-A224 cells pulsed with a corresponding peptide. Backgound IFN-γ in response to the human immunodeficiency virus (HIV) peptide was subtracted. Significant values ($P < 0.05$ by two-tailed Student's t-test) are underlined.

cultured with effector cells in 96-round-well plates at the indicated effector/target ratios. The specific ^{51}Cr-release was calculated according to the following formula: (test c.p.m. – spontaneous c.p.m.) × 100/(total c.p.m. – spontaneous c.p.m.). Spontaneous release was determined by the supernatant of the sample incubated with no effector cells, and the total release was then determined by the supernatant of the sample incubated with 1% Triton X (Wako Pure Chemical Industries, Osaka, Japan). In some experiments, 10 μg/ml of either anti-HLA class I (W6/32: mouse IgG2a), anti-HLA-DR (L243: mouse IgG2a), anti-CD4 (NU-TH/I: mouse IgG1), anti-CD8 (NU-TS/C: mouse IgG2a), or anti-CD14 (H14: mouse IgG2a) mAb was added into wells at the initiation of the culture.

Cold Inhibition Assay

The specificity of PSCA peptide-stimulated PBMCs was confirmed by a cold inhibition assay. In brief, ^{51}Cr-labeled target cells (2×10^3 cells/well) were cultured with the effector cells (2×10^4 cells/well) in 96-round-well plates with 4×10^4 cold target cells. C1R-A24 cells, which were pre-pulsed with either the HIV peptide or a corresponding PSCA peptide, were used as cold target cells.

Detection of Peptide-Specific IgG

Peptide-specific IgG levels in plasma were measured by ELISA as previously reported [19,20]. Briefly, a peptide (20 μg/well)-immobilized plate was blocked with Block Ace (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween-20–PBS, after which 100 μl/well of plasma sample diluted with 0.05% Tween-20–Block Ace was added to the plate. After a 24-hr incubation at 3°C, the plates were washed and further incubated for 2 hr with a 1:1,000-diluted rabbit anti-human IgG (γ-chain-specific) (Dako, Glostrup, Denmark). The plate was washed, and then 100 μl of 1:100-diluted goat anti-rabbit IgG-conjugated horseradish peroxidase (En Vision; Dako) was added to each well, and the plate was incubated at room temperature for 40 min. After the plate was washed again, 100 μl/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was