which are already known; thus expression of these latter antigens in pancreatic cancer cells was highly expected.[11,13,14] For example, cyclophilinB (CypB) is a family of immunophilins involved in the cellular proliferation of both normal and malignant cells,[11] whereas Lck is a family of src proteins also involved in cellular proliferation; the expression of metastatic pancreatic cancers was been reported elsewhere.[13] The other 4 antigens (ppMAPkkk, WHSC2, UBE2V and HNRPL) were cloned from a pancreatic cancer cDNA.[14] All of the 26 peptides used in our study were derived from these 10 tumor-rejection antigens, and all of them were able to induce HLA class I-restricted and tumor-specific CTLs in the PBMCs of cancer patients as reported previously.[9–15]

PBMCs of pancreatic cancer patients (10 HLA-A24$^+$ and 5 HLA-A2$^+$) and healthy donors (5 HLA-A24$^+$ and 5 HLA-A2$^+$) were tested for their reactivity to the 13 peptides for HLA-A24$^+$ patients or the 13 peptides for HLA-A2$^+$ patients, respectively. Representative results of HLA-A24$^+$ and -A2$^+$ subjects are shown in Tables II and III, respectively. The values given in the tables represent the means of quadruplicate assays of IFN-γ production by the peptide-stimulated PBMCs in response to a corresponding peptide. The background IFN-γ production (10–200 pg/ml) in response to an HIV or HTLV-1 peptide was subtracted from the values given in the tables. CTL precursors were judged as positive (the score is double-underlined in the tables) if the mean value of IFN-γ production by the peptide-stimulated PBMCs in response to a corresponding peptide was significantly ($p < 0.05$) higher than that produced in response to an HIV or HTLV-1 peptide. Surface markers of these peptide-induced CTLs were mostly (>70%) CD3$^+$CD4$^-$CD8$^+$ (data not shown). Among 15 patients, 1, 2, 3, 5 and 4 patients had detectable levels of CTL precursors to 5, 3, 2, 1 and 0 peptides of vaccine candidates (Tables II, III). Collectively, peptide-specific CTL precursors were detectable in 11 of 15 (73.3%) of patients, with the mean positive number of 1.5 peptides per patient (ranging from 0–5 peptides). The profile of positive peptides entirely varied among patients. In contrast, CTL precursors reactive to EBV-derived peptides, taken as control peptides to measure patients' immunocompetency, were detectable in only 1 (patient [Pt.] 13) of 15 (6.7%) patients. PBMCs from 4 patients (Pts. 2, 6, 10 and 15) had no detectable levels of CTL precursors reacting to any of the peptides tested.

Peptide-specific CTL precursors were also detectable in 9 of 10 (90%) healthy donors with the mean positive number of 2.0 peptides per donor (range 0–5 peptides) (Tables II, III). CTL precursors reactive to EBV-derived peptides were detectable in 8 of 10 donors (80%). There were no significant differences between cancer patients and healthy donors in regard to reactivity to any of the vaccine candidate peptides. However, the percentages of cases with CTL precursors reactive to EBV peptides in cancer patients were significantly ($p < 0.01$) lower than those in healthy donors.

The peptide-induced CTL activity was confirmed by a 6 hr ^{51}Cr-release assay, and representative results of HLA-A24$^+$ (Pts. 1 and 9) and HLA-A2$^+$ (Pt. 13) patients are shown in Figure 2*a*, *b* and *c*, respectively. The PBMCs of Pt. 1 were stimulated with no peptide, EBV, SART1$_{690}$, SART2$_{161}$ and SART3$_{315}$ peptides and were used as effector cells. The PBMCs contained CTL precursors reactive to the latter 3 peptides as determined by an IFN-γ release assay (Table II). The PBMCs stimulated with SART1$_{690}$, SART2$_{161}$ and SART3$_{315}$ peptides exhibited significant levels of cytotoxicity against HLA class I-matched tumor cells but not against either HLA class I-mismatched tumor cells or HLA class I-matched phytohemagglutinin (PHA)-blastoid T cells (Fig. 2*a*). In contrast, the PBMCs stimulated with no peptide and EBV peptide exhibited only modest levels of cytotoxicity to HLA class I-matched tumor cells and no cytotoxicity to either HLA class

TABLE I – EXPRESSION OF TUMOR-REJECTION ANTIGENS IN PANCREATIC CANCERS (SUMMARY)[1]

Tumor antigens	Pancreatic cancer cell lines		Pancreatic cancer tissues		Nontumorous pancreatic tissues	
	Cytosol	Nucleus	Cytosol	Nucleus	Cytosol	Nucleus
SART1$_{259}$	3/6[2]	0/6	4/7	0/7	0/5	0/5
SART2	7/9	7/9	7/7	5/7	0/5	0/5
SART3	8/9	8/9	5/7	5/7	0/5	0/5
ART4	5/6	6/6	5/7	6/7	0/5	0/5

[1]Expression of SART1$_{259}$, SART2, SART3 and ART4 proteins in pancreatic cancer cells and tissues and nontumorous pancreatic tissues were investigated by Western blot analyses.–[2]Values represent number of positive samples/total number of samples tested.

TABLE II – INDUCTION OF PEPTIDE SPECIFIC CTLS FROM PBMCS OF HLA-A24$^+$ PANCREATIC CANCER PATIENTS[1]

Peptide	Sequence	Pt. 1[2]	Pt. 2	Pt. 3	Pt. 4	Pt. 5	Pt. 6	Pt. 7	Pt. 8	Pt. 9	Pt. 10	HD1	HD2	HD3	HD4	HD5
EBV	TYGPVFMCL	51	49	46	26	0	55	50	10	0	55	235[3]	54	195	266	235
SART1$_{690}$	EYRGFTQDF	72	27	38	0	270	7	70	0	32	0	14	56	0	202	218
SART2$_{93}$	DYSARWNEI	70	15	32	19	0	0	52	44	0	0	0	0	0	159	0
SART2$_{161}$	AYDFLYNYL	95	27	0	0	7	0	54	56	0	0	0	0	0	163	0
SART2$_{899}$	SYTRLFLIL	66	58	0	50	0	0	0	45	31	0	0	0	0	163	0
SART3$_{109}$	VYDYNCHVDL	11	55	0	0	0	0	27	12	20	0	52	104	59	0	0
SART3$_{315}$	AYIDFEMKI	82	5	16	13	50	0	0	43	13	0	37	0	0	76	471
CypB$_{84}$	KFHRVIKDF	0	33	4	1	0	64	93	0	33	0	71	0	86	0	22
CypB$_{91}$	DFMIQGGDF	78	7	0	0	0	9	58	0	50	0	0	0	0	54	0
Lck$_{208}$	HYTNASDGL	25	0	0	0	0	0	99	17	0	0	54	0	4	0	2
Lck$_{486}$	TFDYLRSVL	30	0	6	3	0	6	0	0	0	24	15	0	25	0	1040
Lck$_{488}$	DYLRSVLEDF	50	0	1001	6	46	0	17	0	0	19	534	0	0	0	305
ART4$_{13}$	AFLRHAAL	0	0	33	145	0	25	7	0	0	20	0	0	0	0	94
ART4$_{75}$	DYPSLSATDI	25	26	24	217	38	0	0	0	51	53	62	0	0	0	0

[1]PBMCs (1×10^5 cells/well) were incubated with 10 μM of a peptide in wells of 96-well u-bottom microculture plate in 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (GIBCO BRL), 10% FCS with 100 U/ml of interleukin-2 (IL-2) and 0.1 mM MEM nonessential amino acid solution (GIBCO BRL). 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. Twenty-four hr after the last stimulation, these cells were harvested and tested for their ability to produce IFN-γ in response to C1R-A2402 cells pre-loaded with a corresponding or an irrelevant control (HIV) peptide in HLA-A24$^+$ PBMCs by an ELISA (limit of sensitivity: 10 pg/ml). All experiments were performed by quadruplicate assays. Two-tailed student's *t*-test was employed for statistical analysis. The values given in the Tables represent the mean value of quadruplicate assays of IFN-γ production by the peptide-stimulated PBMCs in response to a corresponding peptide. The background IFN-γ production (10–200 pg/ml) in response to an HIV peptide was subtracted from the values given in the Tables.–[2]Pt., patient; HD, healthy donor.–[3]CTL precursors were judged as positive (the score is double-underlined in the Tables) if the mean value of IFN-γ production by the peptide-stimulated PBMCs in response to a corresponding peptide was significantly ($p < 0.05$) higher than that produced in response to an HIV peptide.

TABLE III – INDUCTION OF PEPTIDE SPECIFIC CTLS FROM PBMCS OF HLA-A2$^+$ PANCREATIC CANCER PATIENTS[1]

Peptide	Sequence	Pt. 11	Pt. 12	Pt. 13	Pt. 14	Pt. 15	HD6	HD7	HD8	HD9	HD10
EBV	GLCTLVAML	6	0	1148	0	0	1002	99	50	60	0
$SART3_{302}$	LLQAEAPRL	32	0	226	8	0	0	0	51	70	0
$SART3_{309}$	RLAEYQAYI	116	0	19	3	27	0	127	30	25	0
$CypB_{129}$	KLKHYGPGWV	43	0	0	0	0	0	0	31	29	6
Lck_{246}	KLVERLGAA	74	16	24	0	0	0	21	29	26	0
Lck_{422}	DVWSFGILL	20	81	10	71	0	0	11	23	60	26
$ppMAPkkk_{294}$	GLLFLHTRT	179	170	0	0	0	0	58	0	0	86
$ppMAPkkk_{432}$	DLLSHAFFA	42	57	58	0	0	0	0	0	0	73
$WHSC2_{103}$	ASLDSDPWV	186	154	0	38	0	0	0	0	0	96
$WHSC2_{141}$	ILGELREKV	0	47	0	659	38	0	0	0	12	260
$UBE2V_{43}$	RLQEWCSVI	0	0	0	7	0	980	31	0	3	56
$UBE2V_{85}$	LIADFLSGL	0	1	13	7	43	0	0	0	0	1226
$HNRPL_{140}$	ALVEFEDVL	0	63	0	317	39	967	0	0	0	99
$HNRPL_{501}$	NVLHFFNAPL	0	0	14	0	13	0	0	0	0	0

[1]Detailed methods for induction of peptide-specific CTLs are shown in a footnote of Table 2 and in Material and Methods. Target cells were T2 cells pre-loaded with a corresponding peptide or an irrelevant control (HTLV-1) peptide.–[2]CTL precursors were judged as positive (the score is double-underlined) if the mean value of IFN-γ production by the peptide-stimulated PBMCs in response to a corresponding peptide was significantly ($p < 0.05$) higher than that produced in response to an HIV peptide.

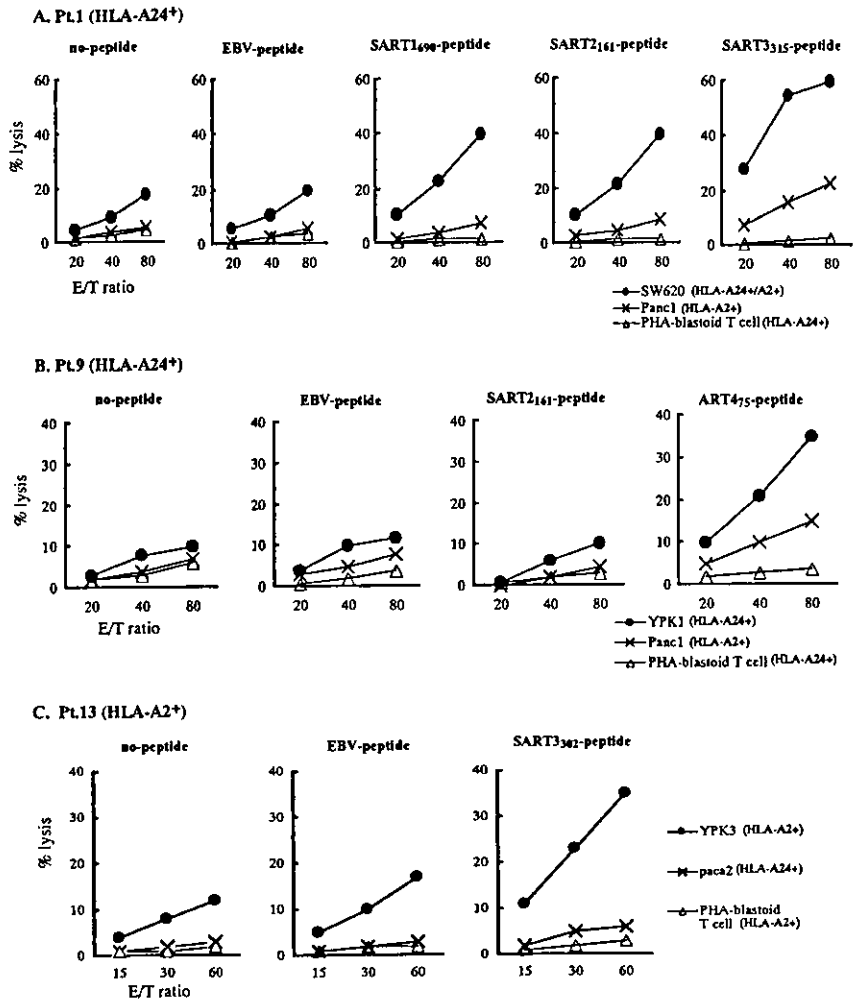

FIGURE 2 – Cytotoxicity against tumor cells. A 6 hr ^{51}Cr-release assay was employed for the measurement of CTL activity of peptide-stimulated PBMCs, and representative results from studies of HLA-A24$^+$ and A2$^+$ patients are shown in Fig. 2*a*, *b* and *c* respectively. EBV peptide-stimulated PBMCs were used as a negative control.

I-mismatched or PHA-blastoid T cells. The PBMCs of Pt. 9 were stimulated with no peptide, EBV, $SART2_{161}$ and $ART4_{75}$ peptides and were used as effector cells. The PBMCs contained CTL precursors reactive to $cyclophilinB_{91}$ and $ART4_{75}$ peptide, but not $SART2_{161}$ peptide, as determined by an IFN-γ release assay (Table II). The PBMCs stimulated with $ART4_{75}$ peptide showed significant levels of cytotoxicity against HLA class I-matched tumor cells but not against either HLA class I-mismatched tumor cells or HLA class I-matched PHA-blastoid T cells (Fig. 2*b*). In contrast, those PBMCs stimulated with no, EBV or $SART2_{161}$

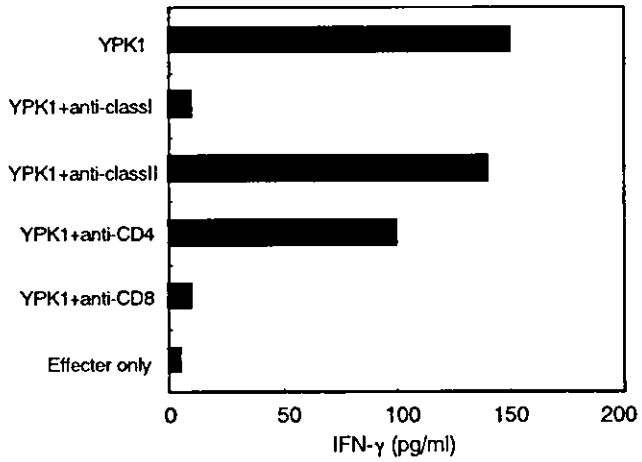

FIGURE 3 – Inhibition of CTL activity. Peptide-stimulated PBMCs of cancer patients were tested for their IFN-γ production by recognition of YPK1 cells (HLA-A24$^+$ pancreatic cancer cells) at an effector to target cell (E/T) ratio of 10/1 in the presence of 20 μg/ml of anti-HLA class I, anti-HLA class II, anti-CD4 and anti-CD8. The result of $ART4_{75}$-specific CTL of Pt. 9 was shown. The values represent the means of triplicate assay. IFN-γ production by effector cells alone was also measured as a negative control.

peptide exhibited only modest levels of cytotoxicity to HLA class I-matched tumor cells and no cytotoxicity to either HLA class I-mismatched tumor cells or HLA class I-matched PHA-blastoid T cells. Similarly, the PBMCs of Pt. 13 have contained CTL precursors reactive to EBV and $SART3_{302}$ peptides as determined by an IFN-γ release assay (Table III). The PBMCs stimulated with $SART3_{302}$ peptide exhibited significantly higher levels of cytotoxicity against HLA class I-matched tumor cells than those stimulated with no peptide or EBV peptide used as a negative control (Fig. 2*c*). None of them exhibited cytotoxicity against HLA class I-mismatched tumor cells or HLA class I-matched PHA-blastoid T cells. These CTL activities were inhibited by anti-class I and -CD8 MAb but not by the other MAbs tested (data not shown). These peptide-specific CTLs showed HLA-A24- or -A2-restricted IFN-γ production against cancer cell lines, and these CTL activities were blocked by anti-HLA class I MAb and anti-CD8 MAb but not by the other MAbs tested (Fig. 3). These results indicate that the peptide-stimulated PBMCs possess MHC class I-restricted and tumor-specific CTL activity. Subsequently, peptides selected by this new culture method could be applicable in use for peptide-based specific immunotherapy for pancreatic cancers.

DISCUSSION

Although peptide-specific CTL precursors were detectable in 73% of the cancer patients studied here (mean positive number: 1.5 peptides), only 26 peptides, 13 for the HLA-A2$^+$ and 13 for the HLA-A24$^+$ cancer patients, were used in our study. Beside these peptides, there are many others with vaccine candidates that possess the ability to induce HLA class I-restricted CTLs reactive to cancer cells.[3–15] Therefore, an increased number of peptides for the assay would be associated with an increased percentage of patients with positive peptides. In addition, an increased number of peptides for the assay would be associated with an increased number of positive peptides per patient. Regardless of these limited conditions, this study showed that peptide-specific CTL precursor cells were detectable in the majority (73%) of HLA-A2$^+$ or -A24$^+$ cancer patients prior to peptide vaccination. These 2 HLA class I alleles are observed in >70% of Caucasians, >80% of Asians and >40% of Blacks.[23,24] All of the 13 peptides for HLA-A2 patients that were used in our study were able to induce CTLs from different subtypes of HLA-A2 (HLA-A0201, 0206 and 0207).[14,15] Therefore, the new method mentioned in our study could be useful to detect peptide-specific CTL precursors in a large number of pancreatic cancer patients. Peptide-specific CTLs induced by the employed method showed HLA-A24- or A2-restricted cytotoxicity against pancreatic cancer cell lines, and these CTL activities were blocked by anti-HLA class I MAb and anti-CD8 MAb. Since the medium containing 10 μM of each peptide was replaced every 3 days in this method, higher concentrations of the peptides could be kept throughout the culture, which in turn might facilitate the generation of HLA class I-restricted and CD8$^+$ CTLs. This assumption is in part due to the fact that large amounts of peptides in culture achieve a high density of peptides on the groove of HLA class I molecule of antigen-presenting cells and therefore tend to stimulate CD8$^+$ CTL as well as T-helper 1 cell responses, whereas low-density presentation tends to elicit T-helper 2 cell responses.

Our study also showed that the profile of positive peptides varied greatly among patients, suggesting that peptides suitable for use in CTL precursor-oriented peptide vaccines are different from patient to patient. These variations would be partly due to 2 factors, namely, heterogeneity of tumor cells and immunologic diversity of T cells in each patient. Subsequently, this new immunotherapeutic approach may be characteristic of an order-made cancer immunotherapy. The same approach might be applicable in the case of malaria or HIV or in other infectious diseases for which no effective vaccine protocols have been established.[21]

CTL precursor frequency analysis is very accurate and sensitive, but it is very laborious, costly and time-consuming and can't handle many samples.[11,13] A recently developed HLA-tetramer assay is also sensitive and accurate.[25] However, it needs both labeled tetramer and a relatively large number of cells per assay, and further, it can't measure CTL activity. The method employed in our study has several demerits from a point of sensitivity and accuracy but could have several merits for monitoring the frequency of the peptide-specific CTLs, including simplicity and the ability to handle relatively large samples. This method can also measure CTL activity.

The frequency of positive peptides with vaccine candidates is not significantly different among cancer patients and healthy donors. This is expected from our previous results showing that all of the tumor-rejection antigens and their peptides used in our study are self-antigens, which are preferentially expressed in proliferating cells, including malignant and normal cells.[8–15] However, CTLs induced by these peptides showed cytotoxicity against cancer cells but not against normal proliferating cells (PHA-blastoid T cells), as demonstrated in our study and also in previous studies.[8–14] Therefore, vaccinations using these peptides may not be associated with adverse effects on normal cells and normal tissues. Indeed, no severe adverse effects were observed in the phase I clinical studies carried out at Kurume University Hospital; these studies analyzed peptide vaccines using 13 different peptides. The same peptides were used *in vitro* in the present study involving HLA-A24$^+$ cancer patients (Gouhara *et al.*, unpublished data).

The frequency of positive cases in cancer patients (1 of 15, 6.7%) who had CTL precursors reactive to EBV peptide was significantly lower ($p < 0.01$) than that of healthy donors (8 of 10, 80%). Further, the PBMCs of 4 patients had no detectable levels of CTL precursors reacting to any of the vaccine candidate peptides, nor to any of the control peptides tested. Cellular immunity of these patients might be depressed, and therefore, the other supportive immunotherapies might be needed for these patients in order to increase general levels of immunity prior to peptide-based specific immunotherapy.

In conclusion, our study investigated CTL precursors in the PBMCs of pancreatic cancer patients prior to vaccination and provided a scientific basis for considering CTL precursor-oriented peptide vaccines as an order-made cancer immunotherapy for the majority of pancreatic cancer patients.

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Gene and Peptide Analyses of Newly Defined Lung Cancer Antigens Recognized by HLA-A2402-restricted Tumor-specific Cytotoxic T Lymphocytes[1]

Akira Yamada,[2] Kouichiro Kawano, Makoto Koga, Shinzo Takamori, Masami Nakagawa, and Kyogo Itoh

Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy [A. Y., K. K., M. K., M. N., K. I.], Department of Immunology [A. Y., K. I.], and Department of Surgery [S. T.], Kurume University School of Medicine, Kurume 830-0011, Japan

ABSTRACT

We investigated tumor antigens recognized by HLA-A2402-restricted CTLs established from T cells infiltrating into lung adenocarcinoma. We report here three newly identified tumor antigen genes, including one unreported gene, temporarily referred to as *clone 83*, and two known genes, *BTB domain containing 2* (*BTBD2*) and *hairpin-binding protein*. These genes were preferentially expressed in most of the cell lines of lung cancer and also of ovarian cancer and renal cell carcinoma at the mRNA level. The expression of these genes was confirmed in lung and other cancer tissue specimens. In normal tissues, *clone 83* was expressed only in the colon, and *hairpin-binding protein* was not expressed at all, whereas *BTBD2* was ubiquitously expressed. *Clone 83*, *BTBD2*, *and hairpin-binding protein* encoded two, one, and one epitope peptides that can be recognized by HLA-A2402-restricted CTLs, respectively. These epitope peptides possessed the ability to induce HLA-A24-restricted tumor-specific CTLs after *in vitro* stimulation in a culture of peripheral blood mononuclear cells from patients with lung cancer. These results suggest that these genes and peptides are potential candidates for cancer vaccines in HLA-A24$^+$ patients with lung cancer.

INTRODUCTION

Lung cancer is among the most commonly occurring malignancies in the world and is one of the few that continues to show an increasing incidence (1). Prognosis of patients with unresectable progressive stages of non-small cell lung cancer is very poor because the response rate to anticancer drugs in these patients is low (1). Therefore, development of new therapeutic modalities for lung cancer is necessary. Specific immunotherapy is one of the most prominent modalities for treatment of these patients. We have previously investigated tumor antigens and their peptides recognized by HLA-A2402-restricted CTLs established from T cells infiltrating into lung cancer and have already identified four lung cancer antigens: cyclophilin B; ART1; ART4; and multidrug resistance-associated protein 3 (2–5). Other tumor antigens reported were also expressed in a substantial number of lung cancers (6–10). Phase I clinical trials using these peptides as cancer vaccines in patients with lung cancer and other cancers are under way at our university hospital (11). However, at present, our clinical trials of peptide-based cancer vaccine, as well as those of other groups, have rarely resulted in tumor regression (11–17). This failure could be attributable to an insufficient induction of CTLs by the relevant peptides that have been identified to date. Therefore, additional studies are needed to resolve this issue and to achieve a better understanding of the molecules involved in host-tumor cell interactions. Alternatively, development of new regimens for therapeutic vaccines might be needed (18). One such regimen could be to consider the group of identified peptide candidates and to choose from among them the most promising vaccine peptides according to a prevaccination measurement of reactivity of memory T cells to the peptides (19). Determination of a relatively large number of candidate peptides for vaccination would allow to choose more suitable vaccine peptides to each patient with different histological types, stages of disease, and immunological levels.

We attempted to clone new tumor antigen genes from lung cancer cells to obtain new candidate peptides in use for vaccination and, here, report three new tumor antigen genes and their derived four peptides recognized by HLA-A2402-restricted CTLs.

MATERIALS AND METHODS

Cell Lines. A lung adenocarcinoma cell line, 11-18, was used for the preparation of a cDNA library. COS7, VA13 (fibroblast), and C1R-A2402 (an HLA-A2402 transfectant, generous gift of Dr. Takiguchi, Kumamoto University, Kumamoto, Japan) cells were used for the transfection and peptide-pulse experiments. The origins and HLA genotypes of cell lines used in this study have been described previously (4, 5).

Cloning and Identification of New Tumor Antigen Genes. The expression-gene cloning method was used to identify genes that encode tumor antigens recognized by the CTL line, GK-CTL, as reported elsewhere (4). The precise methods for preparation of a cDNA library of the 11-18 cells, screening of cDNA clones, and DNA sequencing have been described elsewhere (5). Briefly, mRNA of the 11-18 lung adenocarcinoma cells was converted to cDNA, ligated to *Sal*I adapter, and inserted into the expression vector *pCMV-SPORT2.0* (Life Technologies, Inc., Gaithersburg, MD). A total of 1×10^5 clones from the cDNA library of the 11-18 cells were divided into 1000 wells (the expected number of clones/well was 100) and subjected to the first screening. Purified DNA from the divided pools and 100 ng of *HLA-A2402* cDNA were cotransfected into VA13 cells (1×10^4 cells/well) and analyzed for their activity to stimulate IFN-γ production by the GK-CTLs. Assay protocol is described in the "Peptides and Assays" section. Full-length *HBP*[3] cDNA was cloned into *pCR3.1* (Invitrogen, Carlsbad, CA) by TA-cloning method. A *BTBD2* clone (*KAIA4184*) was purchased from Takara (Tokyo, Japan).

Northern Blot Analysis. Total RNA (5 μg/lane) extracted from various cells or tissue specimens (six lung cancers, two renal cell carcinomas, one colon cancer, four gastric cancers, two ovarian cancers, one esophageal cancer, and one oral cancer) using RNA zol B (TEL-TEST, Friendswood, TX) was separated on formaldehyde-agarose gel and transferred to nylon membranes (Hybond-N$^+$; Amersham, Buckinghamshire, United Kingdom). Total RNA preparations of normal tissues were purchased from Sawady Technology (Tokyo, Japan). The membranes were additionally hybridized with the following probes: *Mlu*I cut 0.7-kb fragments of original *clone 50*; *Pst*I cut 1.2-kb fragments of *clone 83*; *Mlu*I cut 1.2-kb fragments of original *clone 96*; *Mlu*I cut 0.9-kb fragments of original *clone 111*; and *Mlu*I cut 1.6-kb fragments of original *clone 114*. Human β-*actin* cDNA (Clontech, Tokyo, Japan) was also used as a control probe. Hybridization method has been described previously (5).

Peptides and Assays. Synthetic peptides (purity >70%) derived from the deduced amino acid sequence of each of the clones with binding motifs for HLA-A2402 molecules, as described in the literature (20), including motifs of tyrosine or phenylalanine at position 2 and isoleucine, leucine, phenylalanine,

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[2] To whom requests for reprints should be addressed, at Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, Asahi-machi 67, Kurume 830-0011, Japan. Phone: 81-942-31-7744; Fax: 81-942-31-7745; E-mail: akiymd@med.kurume-u.ac.jp.

[3] The abbreviations used are: HBP, hairpin-binding protein; BTBD2, BTB domain containing 2; PBMC, peripheral blood mononuclear cell; nt, nucleotide; ORF, open-reading frame; mAb, monoclonal antibody.

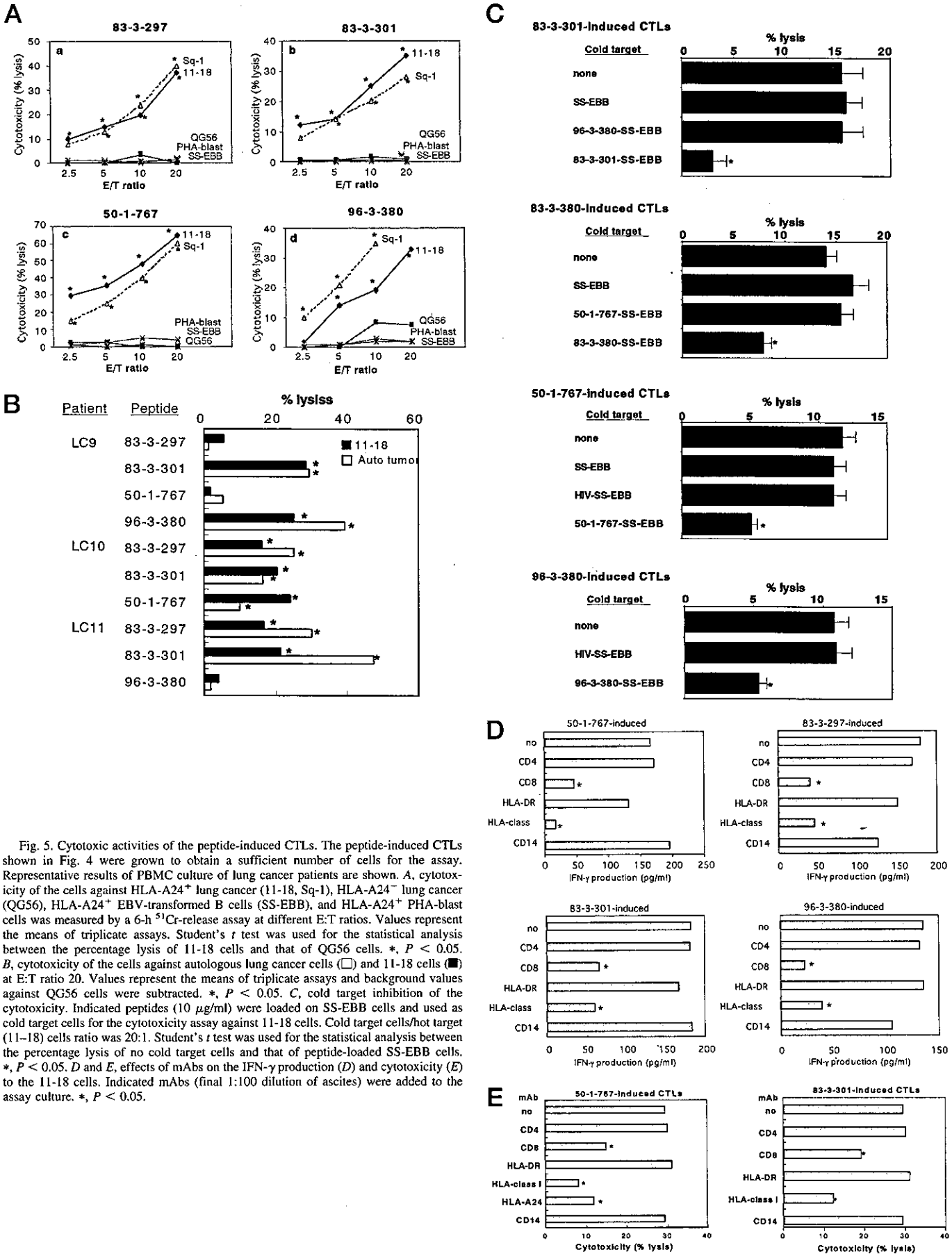

Fig. 5. Cytotoxic activities of the peptide-induced CTLs. The peptide-induced CTLs shown in Fig. 4 were grown to obtain a sufficient number of cells for the assay. Representative results of PBMC culture of lung cancer patients are shown. *A*, cytotoxicity of the cells against HLA-A24$^+$ lung cancer (11-18, Sq-1), HLA-A24$^-$ lung cancer (QG56), HLA-A24$^+$ EBV-transformed B cells (SS-EBB), and HLA-A24$^+$ PHA-blast cells was measured by a 6-h ^{51}Cr-release assay at different E:T ratios. Values represent the means of triplicate assays. Student's *t* test was used for the statistical analysis between the percentage lysis of 11-18 cells and that of QG56 cells. *, $P < 0.05$. *B*, cytotoxicity of the cells against autologous lung cancer cells (□) and 11-18 cells (■) at E:T ratio 20. Values represent the means of triplicate assays and background values against QG56 cells were subtracted. *, $P < 0.05$. *C*, cold target inhibition of the cytotoxicity. Indicated peptides (10 μg/ml) were loaded on SS-EBB cells and used as cold target cells for the cytotoxicity assay against 11-18 cells. Cold target cells/hot target (11–18) cells ratio was 20:1. Student's *t* test was used for the statistical analysis between the percentage lysis of no cold target cells and that of peptide-loaded SS-EBB cells. *, $P < 0.05$. *D* and *E*, effects of mAbs on the IFN-γ production (*D*) and cytotoxicity (*E*) to the 11-18 cells. Indicated mAbs (final 1:100 dilution of ascites) were added to the assay culture. *, $P < 0.05$.

Table 1 *Recognition of gene products of full-length HBP and BTBD2 by CTLs*[a]

Target cells transfected with	Effector cells	IFN-γ (pg/ml)
None	GK-CTLs	27
HLA-A2402 alone	GK-CTLs	34
Clone 96 + HLA-A2402	GK-CTLs	144[b]
HBP + HLA-A2402	GK-CTLs	186[b]
HLA-A2402 alone	50-1-767-induced CTLs	17
Clone 50 + *HLA-A2402*	50-1-767-induced CTLs	79[b]
BTBD2 + *HLA-A2402*	50-1-767-induced CTLs	75[b]

[a] VA13 cells were transfected with the indicated cDNA. Two days after the transfection, cells were co-cultured with effector cells for 18 h, and IFN-γ in the supernatants were measured. Values show mean of triplicate assay.

[b] Values were significantly differed from those of HLA alone controls, $P < 0.05$.

tissues could possibly induce adverse effects such as tissue distraction when the newly defined gene product-derived antigenic peptides are used in treatment vaccines for patients with lung and other cancers. Therefore, *clone83* and *HBP/clone 96* are more suitable target molecules for cancer vaccines. However, it should be noted that no severe adverse effects in normal tissues or organs have been reported in the clinical trials of cancer vaccines specific to MAGE-1, MAGE-3, Melan-A, gp100, tyrosinase, and NY-ESO-1 in melanoma patients, although these molecules are expressed in the normal testis, retina, and/or melanocytes at both mRNA and protein levels (12–17). Similarly, no severe adverse effects on the function of normal organs have been observed in our clinical trials of peptide cancer vaccines, although some of the target molecules are ubiquitously expressed in normal organs (11, 29). Subcellular traffic of antigenic molecules and/or subsequent processing of the antigenic peptides in the proteasomes of normal cells may differ from that of tumor cells in these cases. Alternatively, some molecules in normal cells, including a family of serpins (a group of serine-protease inhibitors), might be involved in normal cell resistance to CTL-mediated lysis (30). Therefore, *BTBD2/clone 50* is also a possible candidate for the cancer vaccine. Study of CTL induction by the *BTBD2/clone 50*-derived peptide also suggests such a possibility, *i.e.,* peptide clone 50-1-767-induced peptide-specific and tumor-specific CTLs in 5 of 8 PBMC cultures from lung cancer patients, whereas no such induction was observed in the PBMCs of healthy donors.

The HLA-A24 allele is found in 60% of Japanese (95% of these cases are genotypically A2402), in 30% of Chinese, and in 20% of Caucasians (31). The four peptides derived from the three genes were able to induce HLA-A24-restricted and tumor-specific CTLs in the PBMCs of lung cancer patients. The four peptides might therefore be appropriate vaccine candidates for use in specific immunotherapy for HLA-A24$^+$ patients with lung cancer.

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The Prostate 57:80 – 92 (2003)

Induction of Cellular and Humoral Immune Responses toTumor Cells and Peptides in HLA-A24 Positive Hormone-Refractory Prostate Cancer Patients by Peptide Vaccination

Masanori Noguchi,[1]* Kazuhiko Kobayashi,[2] Norie Suetsugu,[2] Katsurou Tomiyasu,[1] Shigetaka Suekane,[1] Akira Yamada,[2] Kyogo Itoh,[2] and Shinshi Noda[1]

[1]Department of Urology, Kurume University School of Medicine, Kurume, Japan
[2]Department of Immunology, Kurume University School of Medicine, Kurume, Japan

BACKGROUND. To assess the safety and immune response of a peptide-based immunotherapy for patients with hormone-refractory prostate cancer, a phase I clinical trial was conducted.
METHODS. This study first investigated whether cytotoxic T-lymphocyte (CTL) precursors reacting to peptide with vaccine candidates (14 peptides for HLA-A24 positive patients) were detectable in the pre-vaccination peripheral blood mononuclear cells (PBMCs) of ten patients with hormone-refractory prostate cancer. Patients were then vaccinated subcutaneously with only those peptides to which pre-vaccination PBMCs reacted (CTL precursor-oriented peptide vaccine) for up to four kinds of peptides.
RESULTS. Overall vaccinations were generally well tolerated, but most patients (nine of ten) developed grade 1 local redness and swelling at the injection site. Increased CTL response to both peptides and cancer cells were observed in four of ten patients. Anti-peptide IgG antibodies were also detected in post-vaccination sera of seven of ten patients. One patient achieved a partial response with an 89% decrease in PSA. Stable disease was demonstrated in five of ten patients (50%) for the median duration of 2 months (range, 2–5 months). There were no objective responses of measurable lesions.
CONCLUSIONS. Increase in cellular and humoral immune responses, and decrease in PSA level in some patients support further development of peptide-based immunotherapy for hormone refractory prostate cancer. *Prostate 57: 80–92, 2003.* © 2003 Wiley-Liss, Inc.

KEY WORDS: **phase I study; prostate cancer; immunotherapy; vaccine; CTL**

INTRODUCTION

Hormone refractory-prostate cancer (HRPC) represents the terminal stage in the natural history of prostate cancer and the treatment options have been limited once the hormone refractory stage is reached. The median survival for HRPC patients is approximately 1 year. To date, no agent has been shown to

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

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*Correspondence to: Masanori Noguchi, MD, Department of Urology, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan.
E-mail: noguchi@med.kurume-u.ac.jp

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prolong survival in HRPC patients [1,2]. Novel therapeutic agents for the treatment of HRPC are urgently required.

The discovery of tumor antigens and the identification of their immunodominant epitope have shifted emphasis to the utilization of these epitope to mediate the destruction of growing cancers in humans [3–6]. Many tumor antigens recognized by cytotoxic T lymphocytes (CTLs) have been identified in the past decade [3,4], and specific immunotherapy utilizing their peptides has been under investigation for metastatic melanoma patients [5,6]. New approaches for HRPC with tumor vaccines have also 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 [7–10]. The clinical responses of these trials have been limited but promising.

The progression of prostate cancer from the hormone-sensitive primary to increasingly androgen-independent metastatic lesions is associated with a number of molecular and genetic changes. These changes can affect the expression of specific antigens on the cell surface. Defining the expression of tumor antigens on prostate cancers of different stages is the crucial first step in selecting targets for specific immunotherapy [11–13]. In addition, initiation of immune-boosting through vaccination could be better than that of immune-priming with regard to induction of prompt and strong immunity. If this is also the case for therapeutic vaccines, pre-vaccination measurement of peptide-specific CTL precursors will be important. 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 14 kinds of vaccine candidates with the ability to induce CTLs [14–24], followed by administration of only positive peptides (CTL precursor-oriented peptide vaccine). A phase I clinical trial was conducted to assess the feasibility and tolerance of a peptide-based immunotherapy for patients with HRPC, and we describe in this study the safety, immune responses, and serum prostate-specific antigen (PSA) responses in HLA-A24 positive HRPC patients vaccinated with CTL precursor-oriented peptides.

PATIENTS AND METHODS

Patient Eligibility

Patients with HLA-A24 positive and pathologically confirmed adenocarcinoma of the prostate were entered into the study (Table I). Eligibility required progression of disease after androgen ablation, as defined by at least one of three criteria: two consecutive increasing PSA measurements 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 scan. 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. To control for the effects of androgen ablation, luteinizing hormone-releasing hormone therapy was maintained. All patients previously treated with antiandrogens were required to be off for at least 4 weeks with further evidence of disease progression after cessation of the antiandrogen. Patients were required to wait at least 4 weeks for study entry after the completion of prior chemotherapy,

TABLE I. Patient Characteristics

Patient	Age (years)	Performance status[a]	Clinical stage	Gleason score	Baseline PSA (ng/ml)	Previous treatment[b]	Number of vaccinations received
1	81	0	$T_4N_1M_0$	8	9.3	LHRH, A	5
2	74	0	$T_4N_0M_{1b}$	7	250	LHRH, E	5
3	75	0	$T_4N_0M_{1b}$	7	56	LHRH, E, R	21
4	70	0	$T_4N_0M_{1b}$	7	12	LHRH, A	21
5	85	1	$T_4N_0M_{1b}$	7	68	LHRH, E	10
6	65	0	$T_3N_1M_0$	6	17	P, R, LHRH, E	14
7	62	0	$T_4N_0M_{1b}$	8	18	LHRH, E	6
8	72	0	$T_4N_0M_{1b}$	9	330	LHRH, A, E	7
9	72	0	$T_4N_0M_{1b}$	8	200	LHRH, A	7
10	54	0	$T_4N_0M_{1b}$	7	33	LHRH, A	7

[a]Performance status by EOCG score.
[b]LHRH, luteinizing hormone-releasing hormone therapy; A, antiandrogen; E, estramustine phosphate; R, radiation; P, radical prostatectomy.

radiation therapy, or a change in hormonal therapy. Other criteria included an Eastern Cooperative Oncology Group performance status of 0 or 1, age 85 or lesser, granulocyte 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 hepatitis C were required. Patients with a serious illness or an active secondary malignancy within the prior 5 years were excluded from study entry. Exclusion criteria also included evidence of immunosuppression or autoimmune disease. This study was approved by the Kurume University School of Medicine ethics committee to be conducted according to good research practice guidelines (approved number #2031), and all patients were required to give written informed consent prior to study entry.

Peptide Selection

The peptides used in the present study are listed in Table II. 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-restricted and tumor-specific CTL activity in PBMCs of the cancer patients [14–20]. Before the first vaccination and 7 days after every third vaccination, 30 ml of peripheral blood was obtained, and PBMCs were isolated by means of Ficoll–Conray density gradient centrifugation. A simple method was used to detect peptide-specific CTLs in PBMCs [21–23]. 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 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLLGI) as a negative control in HLA-A24 PBMCs. The target cells (C1R-A2402, 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 by an enzyme-linked immuno-sorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). All experiments were performed in four different wells with duplicate assays. A two-tailed Student *t* test was used for the statistical analyses.

Expression of SART2, SART3, and p56^{lck} in Prostate Tumor

Expression of antigens including SART2, SART3, and p56^{lck} on prostate cell line, PC93, and cancer tissue were analyzed by Western blot analysis and immunostaining. PBMCs and the PHA-stimulated cells (PHA-blast) were obtained from healthy donor. Tumor cell lines used in this study were prostate tumor cell line (PC93) and esophageal cancer cell line (KE4). Prostate cancer tissues samples (n = 7; PT1–PT7) were obtained at the time of surgery in the Kurume University Hospital. A section of each sample was minced with scissors and kept at −80°C until use. The samples were lysed with a buffer consisting of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.2 mM PMSF (Sigma Chemical Co., St. Louis, MO), and 0.03 one trypsin inhibitor unit (TIU)/ml aprotinin, sonicated, and centrifuged at 14,000 rpm for 20 min, and the supernatant was used for SDS–PAGE. The proteins in acrylamide gel were blotted to Immobilon™–polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and were incubated with rabbit anti-SART2 [15], -SART3 [16] antisera, and mouse anti-p56^{lck} monoclonal antibody [Lck (3A5), SANTA CRUZ Biotech., Inc., Santa Cruz, CA], respectively, for 4 hr at room temperature. The other methods of Western blot analysis were previously described [14]. Formalin fixed, paraffin embedded 5.0 μm sections were dewaxed in xylene and rehydrated in graded alcohol. After washing in phosphate buffered saline, 10% normal swine serum was applied for 20 min to block nonspecific staining. After draining, slides were incubated for 60 min with mouse anti-p56^{lck} monoclonal antibody [Lck(3A5), SANTA CRUZ Biotech., Inc.]. A biotinylated secondary antibody was layered with a horseradish peroxidase-strepavidin conjugate. Bound antibody was visualized after red color development using 3-amino-9-ethylcarbazole (AEC).

Vaccination Schedule

For the skin test, 10 μg of each selected peptide for up to five 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 >10-mm diameter erythema and induration, when saline was a negative control for assessment of the hypersensitivity. If immediate-type

TABLE II. Vaccinated Peptides, Immune Responses, and Clinical Response (Summary)

Case (patient number)	Peptide	Sequence	Cellular response to peptide[a]		Anti-peptide Ig G[b]		DTH induction	Increased cytotoxicity[c]	% Reduction in PSA	Best clinical response
			Pre	Post (6)	Pre	Post (6)				
1 (006)	SART $3_{109-118}$	VYDYNCHVDL	E	n.a.[e]	–	+ (3)[d]	–	–	53 (2)	SD
	SART $3_{315-323}$	AYIDFEMKI	E	n.a.	–	–	–			
	$lck_{208-216}$	HYTNASDGL	E	n.a.	–	–	–			
	$lck_{488-497}$	DYLRSVLEDF	E	n.a.	–	–	–			
2 (008)	SART 2_{93-101}	DYSARWNEI	EE	n.a.	–	–	–	–	None	PD
	SART $2_{161-169}$	AYDFLYNYL	C	n.a.	–	–	–			
	SART $2_{899-907}$	SYTRLFLIL	AD	n.a.	–	–	+ (3)			
	SART $3_{109-118}$	VYDYNCHVDL	D	n.a.	–	–	–			
3 (010)	ART $1_{188-196}$	EYCLKFTKL	ArAC	A	–	++(6)	–	–	None	PD
	ART 4_{75-84}	DYPSLSATDI	AE	E	–	–	–			
	CyB_{84-92}	DFMIQGGDF	A	–	+	+ (3)	–			
	$lck_{208-216}$	HYTNASDGL	AD	–	–	+ (6)	–			
4 (014)	ART 4_{75-84}	DYPSLSATDI	B	–	–	–	–	+ (9)	None	PD
	SART 2_{93-101}	DYSARWNEI	ArB	–	–	–	–			
	SART $3_{109-118}$	VYDYNCHVDL	ArB	ArAA	–	+ (6)	–			
	SART $3_{315-323}$	AYIDFEMKI	C	AC	–	–	–			
5 (015)	SART $1_{690-689}$	EYRGFTQDF	AAE	E	–	–	–	+ (3)	16 (3)	SD
	SART $2_{161-169}$	AYDFLYNYL	A	–	–	–	–			
	$lck_{208-216}$	HYTNASDGL	C	AAC	–	–	–			
	$lck_{486-494}$	TFDYLRSVL	C	AC	–	–	+ (5)			
6 (016)	ART $1_{188-196}$	EYCLKFTKL	AE	–	–	++ (6)	–	–	None	SD
	SART $1_{690-689}$	EYRGFTQDF	C	–	–	–	–			
7 (017)	ART $1_{188-196}$	EYCLKFTKL	ArAA	–	–	++ (6)	–	–	None	PD
	SART $3_{109-118}$	VYDYNCHVDL	A	A	–	+ (5)	–			
	SART $3_{315-323}$	AYIDFEMKI	ArA	A	–	–	+ (4)			
	$lck_{488-497}$	DYLRSVLEDF	ArAE	–	–	–	+ (4)			
8 (019)	SART $1_{690-689}$	EYRGFTQDF	BE	–	–	–	–	+ (3)	5 (2)	SD
	SART $2_{899-907}$	SYTRLFLIL	AAD	A	–	–	–			
	SART $3_{109-118}$	VYDYNCHVDL	AD	ArArArA	–	–	–			
	$lck_{486-494}$	TFDYLRSVL	CD	–	–	+ (6)	+ (5)			
9 (020)	SART 2_{93-101}	DYSARWNEI	Ar	–	–	–	–	+ (3)	89 (3)	PR
	CyB_{91-99}	DFMIQGGDF	C	CE	–	–	–			
	$lck_{208-216}$	HYTNASDGL	AD	E	–	–	+ (6)			
	$lck_{486-494}$	TFDYLRSVL	A	AA	–	–	+ (6)			

10 (021)	SART 2_{93-101}	DYSARWNEI	A	–	–	+ (6)	–	–	33 (2)	SD
	SART $2_{161-169}$	AYDFLYNYL	Ar	A	–	++ (6)	+ (7)			
	$lck_{486-494}$	TFDYLRSVL	CD	–	–	–	–			

*P value (Student's t test), **specific IFN-γ production (pg/ml) was calculated by subtracting the response to HIV-derived irrelent peptide. AAAA: all four wells of quaricate assay were positive. AAA: three wells of quaricate assay were positive. AA: two wells of quaricate assay were positive. A: one well of quaricate assay was positive.

[a]The CTL precusor assay was performed and was each well evaluated by the following criteria, and up to four peptides were administrated; Ar: $P^* \leq 0.1$ and $500 \leq$ net**; 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; D: $0.05 < P \leq 0.1$, and $25 \leq$ net ≤ 50; E: $0.1 < P \leq 0.3$ and $100 \leq$ net.

[b]Number of the vaccination when IgG to the peptide was detected for the first time.

[c]Cytotoxicity to *HLA-A2402* gene transfected PC93 cells by IL-2 stimulated PBMCs of the pre- and post-vaccination (six times) was tested by standard 6β hr+Cr-release assay at E/T ratio of 10/1. Cytotoxicity to *HLA-A2402* gene transfected PC93 cells was subtracted from the values. Values represent the means of the triplicate assay.

[d]–, absent; +, present, low titer; ++, present, high titer.

[e]Not available.

hypersensitivity was negative, the peptide was injected. Three milliliters of each of the peptide solutions at 3 mg/ml were mixed with an equal volume of the IFA (Montanide ISA-51; Seppic, Paris, France) and emulsified in the 5-ml plastic syringes, and 3 ml of each of the peptides injected subcutaneously in the lateral thigh at 2-week intervals. Patients were observed until objective disease progression or intolerance, or withdrew consent. Serum PSA levels were measured every 4 weeks during vaccinations. Time to progression was defined as the time from the day of registration until the day objective disease progression was documented. Patients who elected to come off the study without objective disease progression (e.g., for increasing PSA) were considered to have disease progression at the time of study withdrawal.

Evaluation of Immune Response

To study immune responses, 30 ml of peripheral blood was withdrawn prior to the first vaccination and 7 days after every third vaccination, and PBMCs were isolated and cryopreserved at −196°C before use. Cytotoxic activity was measured using a standard 6 hr ^{51}Cr-relase assay, as reported previously [24]. In brief, cryopreserved PBMCs were thawed, and cultured in the medium consisting of 45% RPMI-1640 medium, 45% AIM-V® medium (Invitrogen Corp.), 10% FCS, 100 U/ml of interleukin-2 (IL-2), and MEM nonessential amino acid solution (Invitrogen Corp.). On the 14–21 days of culture, the cells were harvested and served for the assay. To avoid the bias of bioassays, PBMCs harvested at different times from a single patient were thawed at the same time. The target tumor cell lines used in this study were, *HLA-A2402* gene transfected C1R (C1R-A2402) cells, PC93 prostate cancer cell line, and *HLA-A2402* gene transfected PC93 prostate (PC93-A2402) as reported previously [25]. An ELISA was used to detect the serum IgG levels specific for the peptides administrated, as reported previously [24]. To estimate peptide-specific IgG levels, the optical density values of each sample were compared with those of serially diluted standard samples, and the values were shown as optical density.

Clinical Evaluation and Response Criteria

Pretreatment evaluation included a history and physical examination with assessment of performance status, and laboratory studies including complete blood count, serum chemistry profile, PSA level, chest, radionuclide bone scan, computed tomography (CT) of the abdomen and electrolytes, and chest film. Complete blood counts, chemistry profiles, and PSA assessments were repeated every 4 weeks. Bone scan and CT scan were repeated every 3 months if positive at baseline.

Toxicity was assessed at least every 2 weeks using the National Cancer Institute common toxicity criteria. Overall response for the study was based on PSA response, response of measurable lesions, and response of nontarget lesions using consensus criteria (response evaluation criteria in the Solid Tumors Group and PSA Working Group) [26,27]. For the PSA response, a complete response (CR) was defined as normalization to <0.2 ng/ml on at least two PSA tests maintained for at least 4 weeks. A partial response (PR) was defined as a 50% decrease maintained for at least 4 weeks, and progression (PD) was defined as an increase of 25% above the nadir. For a measurable disease response, a CR is defined as disappearance of all target lesions for at least 4 weeks. A PR was defined as a decrease of at least 30% in the sum of the longest diameters of target lesions maintained for at least 4 weeks. PD was defined as an increase of at least 20% in the sum of the longest diameters of target lesions or the appearance of new lesions. For the overall response, a CR requires CR of both target and nontarget lesions in addition to normalization of PSA levels. An overall PR requires a CR or PR of both target lesions and PSA, and non-PD of nontarget lesions. PD of target lesions, nontarget lesions, or PSA or development of new lesions constitutes overall PD.

RESULTS

Expression of SART2, SART3, and p56^{lck} in Prostate Cancer

Expression of SART2, SRAT3, and p56^{lck} antigens in prostate cancer cell line and cancer tissues was analyzed at the protein levels by Western blot analysis. Representative results are shown in Figure 1. SART2 and SART3 antigens were detectable in the KE4 esophageal cancer cell line, a positive control from which the antigens were cloned, PC93 prostate cancer cell line, and four of seven prostate cancer tissues. In contrast, these antigens were not detectable in PBMCs, and/or MKN45 gastric cancer cell line as negative controls, and also in the remaining three prostate cancer tissues. p56^{lck} antigen, a family of src proteins involved in proliferation, was expressed in PBMCs, PHA-blasts, and KE4 tumor cells, as positive controls [18]. It was expressed in the PC93 and four of seven prostate cancer tissues. A few, but significant numbers of prostate cancer cells in cancer tissue samples were also positive by means of immunohistological staining (data not shown). These results are consistent with our previous results regarding other cancers as reported [15,16,18]. None of them were expressed in nontumorous prostate tissues (data not shown). Antibodies to the other four antigens (SART1, CypB, ART-1, and ART-4) were not available at the present time, and their expression in

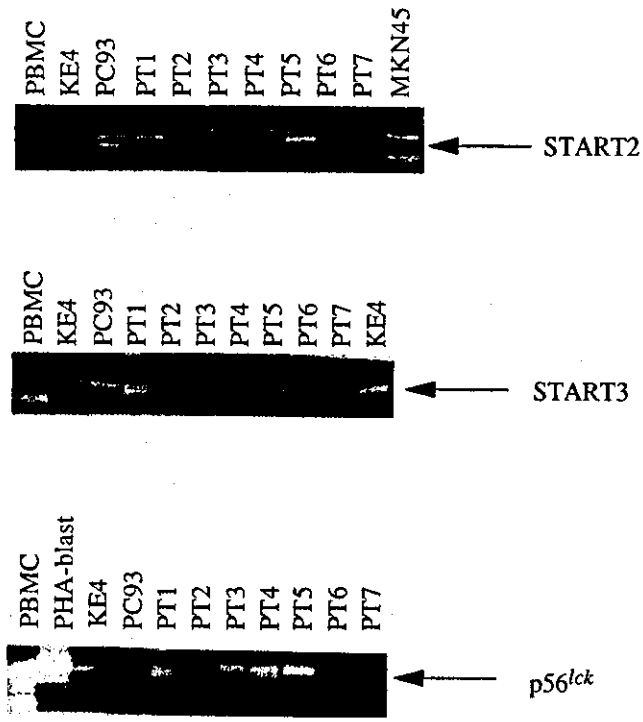

Fig. 1. Expressions of SART2 **(top)**, SART3 **(middle)** and p56^{lck} **(bottom)** by Western blot analysis. The samples shown in the figure are normal cell (PBMCs), PHA-blast, two tumor cell lines (KE4 and PC93), and seven fresh prostate tumor tissues (PT1-PT7). PBMC and MKN45 were used as negative and KE4 was used as positive controls for SART2, respectively. The 100-kDa band of SART2 (the band showed by arrow at top of the figure) was detectable in KE4, PC93, PT1, PT3, PT4, and PT5. PBMC and KE4 were negative and positive controls of SART3, respectively. The 140-kDa band of SART3 (the band showed by arrow at middle of the figure) was detectable in KE4, PC93, PT1, PT3, PT4, and PT5. The 56-kDa band of p56^{lck} (the band showed by arrow at bottom of the figure) was detectable in KE4, PC93, PT1, PT3, PT4, and PT5. PBMC and PHA-blastoid cells were used as a positive control of p56^{lck}.

prostate cancers. Ten were not studied. However, all of them were nonmutated self-antigens preferentially expressed in proliferating malignant cells as reported previously [14,17,19,20,23], and thus expression of these antigens in prostate cancer cells was highly expressed. For example cyclophilin B (CypB) is a family of immunophilins involved in cellular proliferation of both normal and malignant cells [17].

Patient Demographics

Ten patients with hormone-refractory prostate cancer were enrolled in this study. The characteristics of the patients are summarized in Table I. The median age of the patients was 72 years (range, 54–85 years) and median Eastern Cooperative Oncology Group performance status was 0 (range; 0–1). All patients had a

rise in PSA (median, 44.5 ng/ml; range, 9.3–330 ng/ml) after previous treatment including hormone therapy, radiation, and radical prostatectomy. At study entry, eight patients had metastatic disease to the bone, and two patients had enlarged pelvic nodes on CT scan but unremarkable bone scans.

Screening of Peptide-Specific CTL-Precursors

Pre-vaccination PBMCs were provided for screening of the CTL precursors reactive to 14 peptides for different wells with duplicate assays in each well, and the results of each well were classified into seven groups in accordance with the P values (by two-tailed Students' t-test) and the amounts of IFN-γ (a mean value response to a corresponding peptide minus that to a 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; net; D: $0.05 < P \leq 0.1$ and $25 \leq$ net ≤ 50; E: $0.1 < P \leq 0.3$ and $100 \geq$ net. Peptides were chosen based upon evaluation of all four wells by the order shown above. Two representative examples are shown in Figure 2. As a result, peptide-specific CTL precursors were detectable in all ten patients, with a median positive number of four peptides (range, 2–5 peptides) per patient (Table II). Each of these candidate peptides was used for an immediate hypersensitivity test, and if negative, the peptides for up to four peptides were vaccinated subcutaneously based on the orders method above. The fifth peptide was vaccinated if some peptides from the first to fourth peptides elicited immediate hypersensitivity. As a result, the most frequently selected peptide in the first screening was $SART2_{93-101}$ (4/10) following $ART1_{188-196}$ (3/10), SART $2_{899-907}$ (3/10), $SART3_{109-118}$ (3/10), and $lck_{208-216}$ (3/10).

Treatment and Adverse Events

The median number of vaccinations to the patients was seven times (range; 5–21 times). Two patients stopped the vaccination because of the disease progression to death: one patient died from renal failure due to rapidly growing tumor in the prostate, and the other patient died from brain bleeding due to bone marrow suppression by the bone metastases. The remaining eight patients continued the vaccination until the end of the study. All 10 patients were evaluated for all common toxicities, and the overall toxicities are shown in Table III. The overall vaccinations were generally well tolerated, but most patients (9 of 10) developed grade 1 local redness and swelling at the injection site. Four patients developed grade 2 hematuria, two patients complained of fatigue, one patient developed grade 1 fever, and another one patient developed grade 1 diarrhea.

Immune Responses

Vaccinated peptides, immune responses and clinical response are summarized in Table II. Immediate type hypersensitivity to one of the chosen peptides was observed in skin tests prior to the first vaccination in 5 of 10 patients, while it was not observed in the remaining five patients (data not shown). Peptides with the ability of eliciting immediate type hypersensitivity were mainly $CypB_{84-92}$ and $ART_{413-420}$ as reported previously [29]. Peptide-specific DTH reactions were observed in six patients after vaccination (Table II). It is of note that the four (cases 5, 8–10) of six patients who demonstrated a positive skin test for DTH reactions experienced a reduction in PSA levels after vaccinations, whereas only one patient (case 1) who remained skin test negative demonstrated a reduction in PSA (Table II).

Increased levels of cellular immune responses to both the vaccinated peptides and HLA-A24-positive prostate cancer cells were obtained in post-vaccination PBMCs of 4 of 8 and 4 of 10 patients tested, as evaluated by both a semi-quantitative CTL-precursor assay based on IFN-γ production and a standard 6-hr ^{51}Cr release assay, respectively. Cellular responses to the vaccinated peptides increased in post-vaccination PBMCs of case 4 (two SART3 peptides), case 5 (two lck peptides), case 8 ($SART3_{109-118}$), and case 9 ($CypB_{91-99}$, $lck_{486-494}$) (Table II and Fig. 2). No such increase was detected in post-vaccination PBMCs from the remaining four patients. CTL activity against prostate cancer cells in pre- and post-vaccination PBMCs is shown in Figure 3. Significant increase of HLA-A24-restricted cyototxicity against prostate cancer cells was observed in post-vaccination PBMCs of four of eight cases tested (cases 4, 5, 7, and 9) as follows: case 4 after the 9th vaccination; case 5 after the 3rd and 6th vaccination; case 7 after the 3rd vaccination; and case 9 after the 3rd vaccination (Fig. 3).

Anti-peptide IgG was not detectable in pre-vaccination sera from most patients (9 of 10), but low levels of anti-$CypB_{84-92}$ was detectable not only post-vaccination but also in pre-vaccination serum from only one patient (case 3) (Fig. 4, top and left column). The levels of IgG antibodies against corresponding vaccinated peptides were induced in post-vaccination sera of 7 of 10 patients as follows: in case 1 (anti-$SART3_{109-118}$), case 3 (anti-$ART1_{188-196}$, $lck_{208-216}$), case 4 (anti-$SART3_{109-118}$), case 6 (anti- $ART1_{188-196}$), case 7 ($ART1_{188-196}$, anti-$SART3_{109-118}$), case 8 (anti-$lck_{486-494}$), and case10 (anti-$SART1_{93-101}$, and anti-$SART2_{161-169}$). Representative results are shown in Figure 4, and the summary is given in Table II. Significant levels of anti-$ART1_{188-196}$ and anti-$SART3_{109-118}$ IgG became detectable in the post-vaccination sera of 3 (cases 3, 6, and 7)

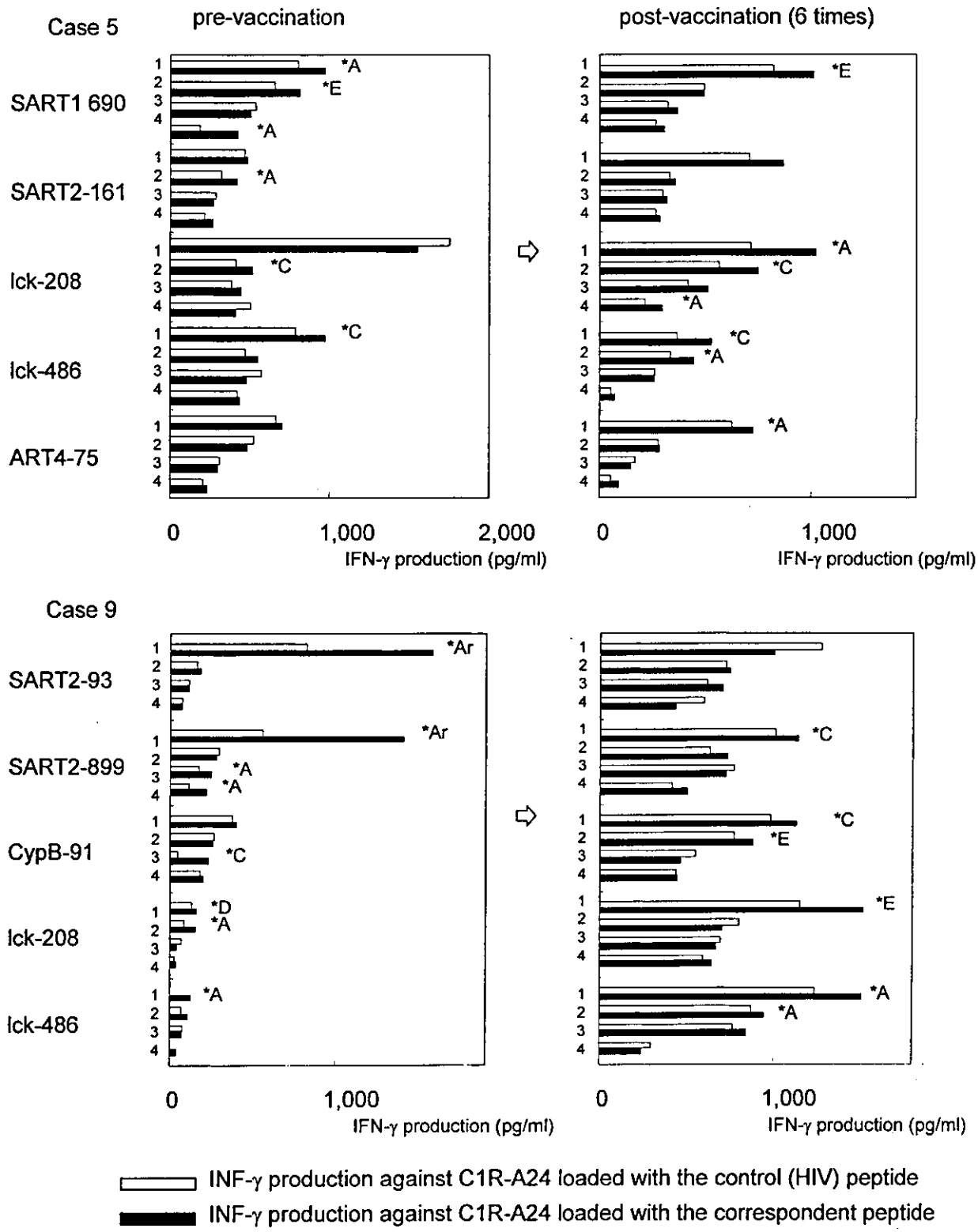

Fig. 2. Kinetics of peptide-specific CTL precursors. PBMCs of either pre- or post-vaccination (six times) from cases 5 and 9 were tested for their reactivity to a corresponding peptide by the methods, as described in Materials and Methods. Values represent the means of four wells and each well was evaluated based on the following criteria. Ar, $P \leq 0.1$ (by two-tailed Students'*t*-test) and $500 \leq$ net value (the amount of IFNγ in response to the corresponding peptide minus that of in response to HIV peptide); A, $P \leq 0.05$ and $50 \leq$ net value; B, $P \leq 0.05$ and $25 \leq$ net value < 50; C, $0.05 < P \leq 0.1$ and $50 \leq$ net value; D, $0.05 < P \leq 0.1$ and $25 \leq$ net value ≤ 50; E, $0.1 < P \leq 0.2$ and $100 \leq$ net value. $SART2_{899-907}$ peptide was not vaccinated for a case 9 because of the appearance of immediate-type hypersensitivity by pre-vaccination test.

TABLE III. Adverse Events of Vaccination

	Grade[a]				
Toxicity	1	2	3	4	Total
Dermatologic	9				9
Hematuria		4			4
Fever	1				1
Fatigue	2				2
Diarrhea	1				1

[a]Toxicities based on the National Cancer Institute common toxicity scale.

and 3 (cases 1, 4, 5) peptides, respectively. The specificity of there IgG was confirmed by inhibition tests with soluble peptides (Fig. 5). The binding of each peptide-specific antibody in the sera of patients was inhibited by the addition of a corresponding peptide, but not by irrelevant peptides in ELISA. A part of the representative results of inhibition tests are shown in Figure 5.

Clinical Responses

Ten eligible patients were evaluable for clinical responses. Only one patient achieved a PR with an 89% decrease in PSA. Figure 6 depicts PSA levels of this patient throughout the study and observation period. Stable disease was demonstrated in 5 of 10 patients (50%) for the median duration of 2 months (range, 2–5 months). There were no objective responses of measurable lesions. The median follow-up duration at the time of this report was 7 months (range; 3–12 months). Two patients had died, and the median survival has not been reached. The eight other patients were alive at 3–12 months after starting the vaccination, and all were treated as out-patients, and their quality of lives were quite good.

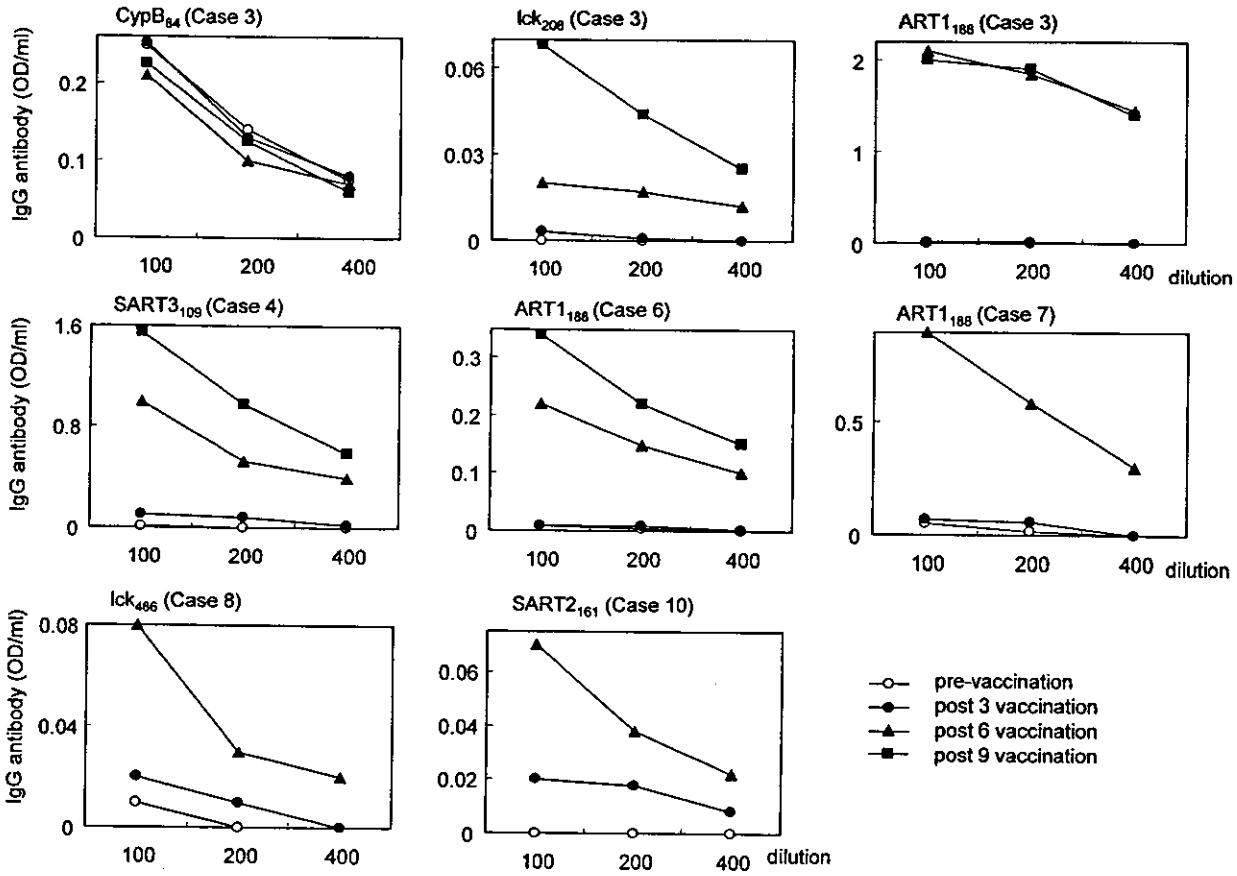

Fig. 3. CTL activity. Pre- or post-vaccination PBMCs were incubated in vitro with IL-2 alone for 14–21 days, as described in Materials and Methods, and their cytotoxicity to the parental PC93 (HLA-A24 negative prostate cancer cells), PC93-A24 (HLA-A24 transfected PC93 cells), and PHA-activated T cell blasts (HLA-A24 positive) were tested by a 6-hr ^{51}Cr-release assay at an E/T ratio of 20/1. Values are the means of triplicate wells. *$P < 0.05$ by the two-tailed Student's *t*-test between the level of the % lysis against PC93-A24 (closed circle) and against PC93 (open circle).

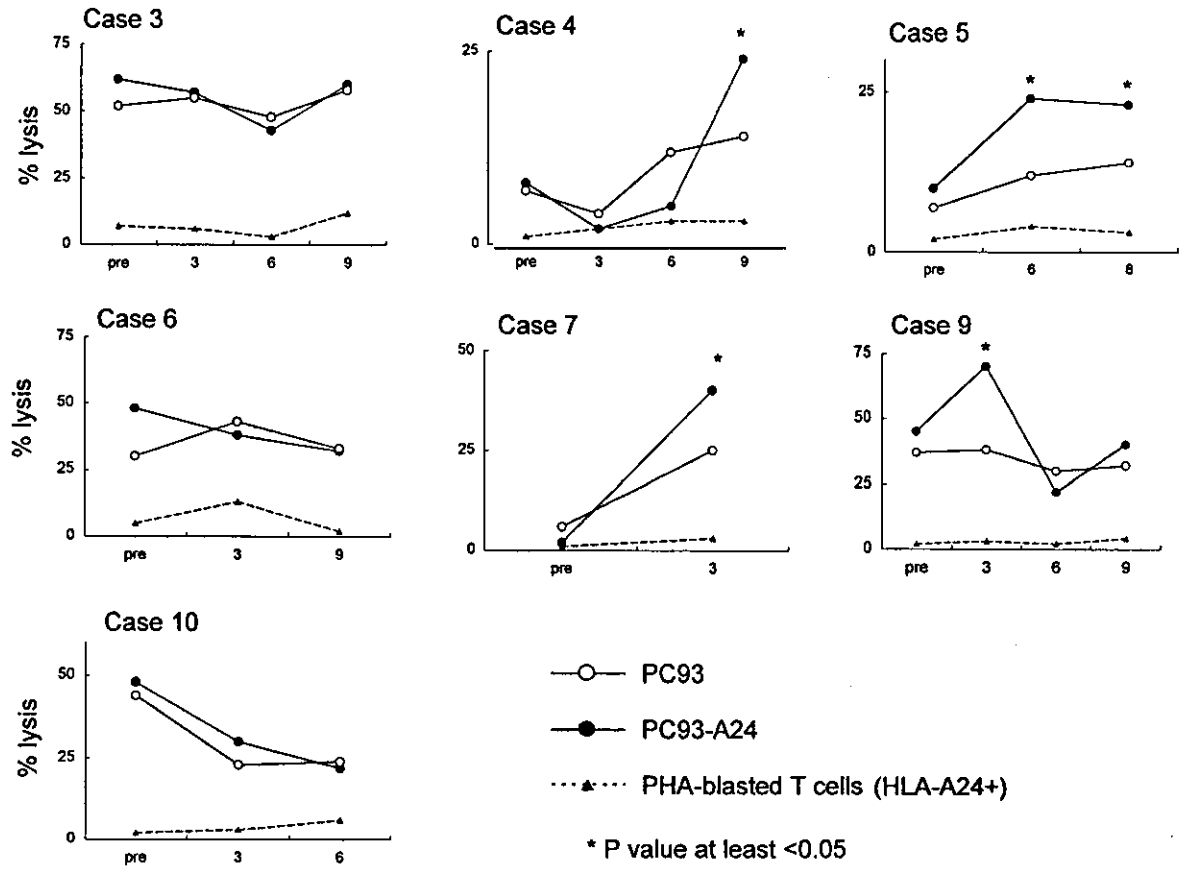

Fig. 4. Peptide-specific IgG in sera of patients. Pre- and post-vaccination sera were serially diluted and the levels of peptide-specific IgG were measured by ELISA, as described in Materials and Methods. Horizontal lines indicated O.D./ml and vertical lines indicate dilution of sera.

DISCUSSION

In this study, we examined the safe administration of a CTL precursor-oriented peptide vaccination using up to four peptides from 14 vaccine candidates to HLA-A24-positive 10 patients with advanced HRPC, most of whom (8 of 10) had bone metastasis. There were no severe adverse effects, but most patients (9 of 10) developed grade 1 local redness and swelling at the injection site. The previous peptide vaccine trials for melanoma patients showed no obvious dose-dependent increases in cellular responses among 0.1–10 mg/injection regimens [5,6,30,31], and subsequent treatment of 1–3 mg/injection at 2–4-week intervals is usually recommended in such clinical trials. In addition, our previous study showed that highest responses were found in patients with 3 mg/injection of peptides, as evaluated by both a standard 6-hr ^{51}Cr release assay and CTL precursor frequency analysis [24]. Therefore, we used 3 mg/injection of peptides and 2-week intervals in this study.

The 14 peptides used in this study had the ability to induce HLA-A24-restricted and tumor specific CTL in cancer patients [14–20]. Zhang et al. [11–13] screened for the expression of 30 different potential prostate cancer cell surface antigens to identify the nine most widely expressed antigens. However, none of these nine were strongly expressed on each prostate cancer cell, suggesting the need for a polyvalent vaccine or mixture of monoclonal antibodies. This study showed that the three antigens, from which peptides used for vaccination were derived, were expressed in the majority of prostate cancers at the protein levels. The present study also showed that the profile of positive peptides greatly varied among patients. All these results suggest that peptides suitable for use in vaccination are different from patient to patient, mainly dependent on large diversity of TCR repertoire.

The present approach in the immunotherapy for HRPC patients took 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 positive for pre-vaccination measurement among 14 vaccine candidates. The results showed that peptide-specific CTL precursors were detectable in 9 of 10 patients prior to peptide vaccination, and also showed that significant levels of increase in cellular immune responses to both

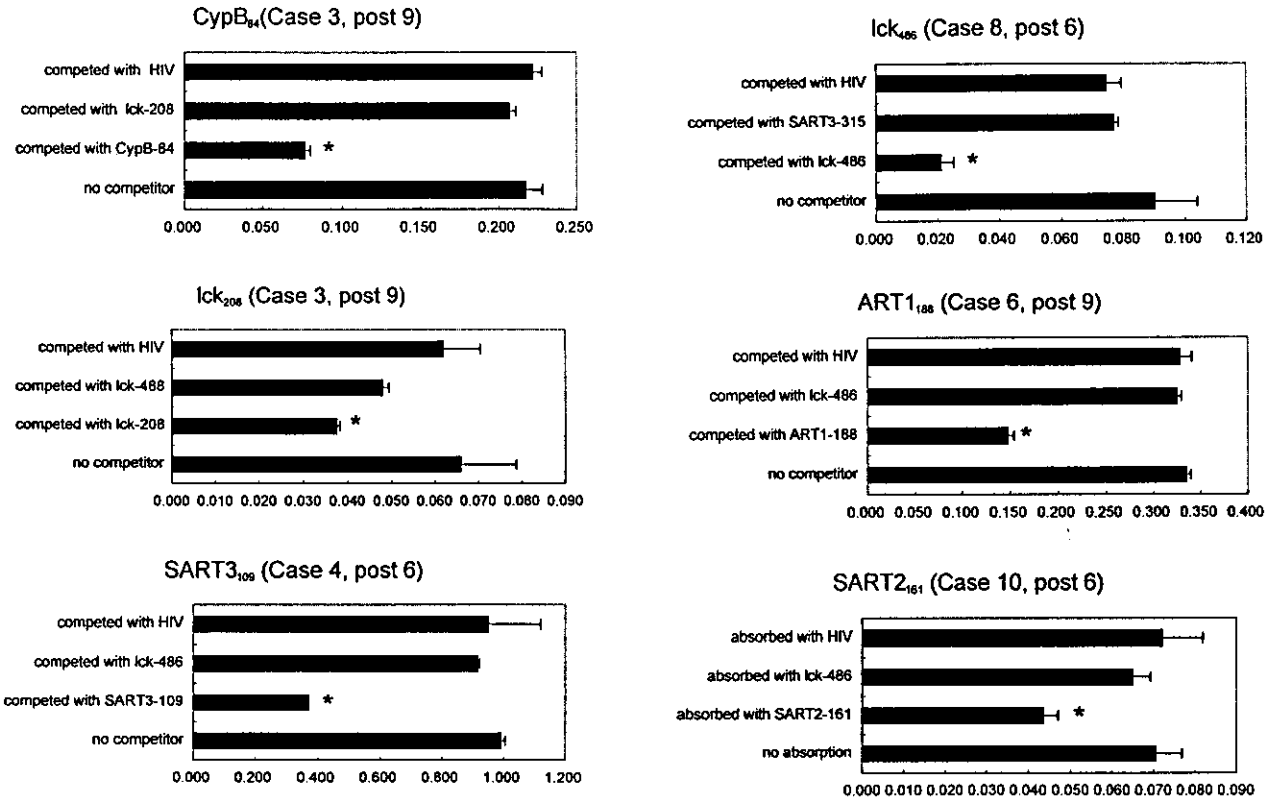

Fig. 5. Inhibition tests of peptide-reactive IgG. Values are the means of triplicate assays. $*P < 0.05$ by the two-tailed Student's *t*-test between the level of the IgG that were adsorbed with and a corresponding peptide or irrelevant peptides which were HIV peptide or irrelevant peptides. Representative cases were shown in this figure.

HLA-A24-positive prostate cancer cells and the vaccinated peptides were obtained in 4 of 8 and 4 of the 10 patients tested, respectively. Anti-peptides antibody was induced in post-vaccination sera of most cases. Because of induction of specific antibodies against these vaccinated peptides, such HLA-class-I restricted peptides seem to have the potential to enhance T helper cell responses in vaccinated hosts, as reported previously [24]. All these results suggest that more prompt and stronger immune responses to both the vaccinated peptides and tumor cells were induced in patients entering to this new protocol as compared to patients entering to the conventional vaccination protocols in which pre-vaccination measurement of peptide-specific CTL precursors in the circulation was not carried out [24,29]. Failure to detect cellular responses to any vaccinated peptides in four of eight patients and also in the remaining four patients whose post-vaccination PBMCs responded to a part of the vaccinated peptides could be in part due to the lower sensitivity of this assay as reported previously [21]. Alternatively, loss of HLA-A24 expression in prostate cancer cells could be involved in this failure [32].

There are several advantages of the monitoring of peptide specific antibodies compared with those of cellular responses. One of them could be high sensitivity. Namely, anti-peptide-IgG became detectable in sera of seven out of ten patients, while cellular responses to peptides were detectable in PBMCs of four out of eight patients. The others could include high reproducibility and simplicity of the method, and time-saving. IgG molecules against these peptides in sera from patients were stable and the method to detect these antibodies was very simple, while detection of CTL responses in PBMCs was not always stable and the method was relatively complicated. Measurement of IgG takes 2 days, while that of cellular responses takes 14 days. However, the roles of up-regulated individual antibodies from immunological and clinical points of view were not clear at the present time.

Although T cell-mediated activity is the predominant pathway of anti-tumor responses, it is well accepted that humoral immune responses also play an important role in tumor immunity. A strong correlation was observed between the humoral responses and overall survival in one clinical study [33]. In that study of stage III melanoma patients, repeated immunization with a polyvalent, allogeneic melanoma cell vaccine generated greatly elevated titers of IgG and IgM recognizing a 90-kDa glycoprotein melanoma-associated

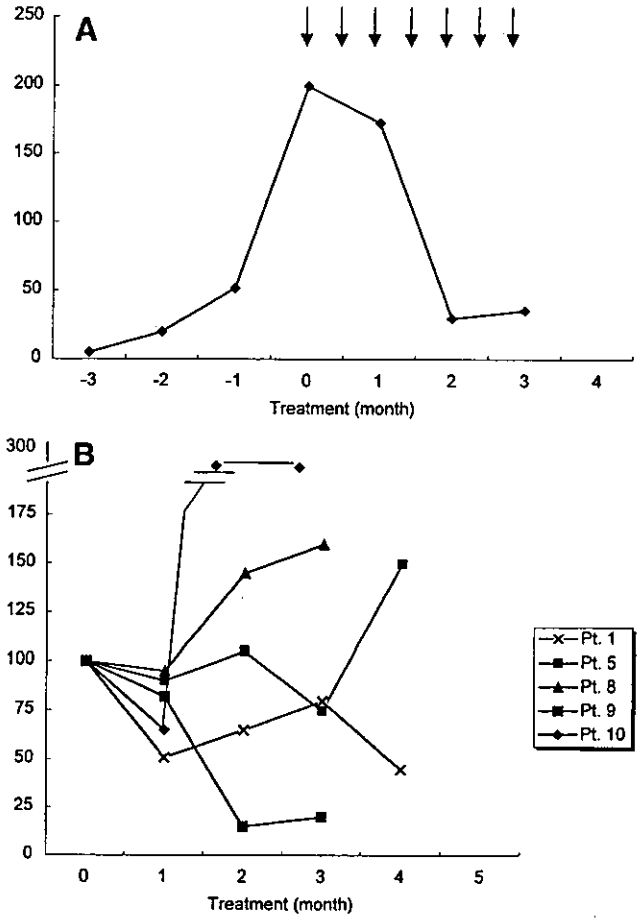

Fig. 6. Measurement of PSA levels during vaccination. **A**: Profile of serum PSA in one patient who experienced an 89% decrease in PSA after vaccination. Arrows indicate time points at which peptides were vaccinated. **B**: PSA response during treatment with the peptide vaccines, expressed as a percentage of pretreatment PSA. Five of 10 patients showed a PSA reduction after vaccination, while the remaining five patients did not show any PSA reduction.

antigen. In the present study, we observed anti-peptide IgG antibody in post-vaccination sera of seven of ten patients. However, we could not find a correlation between the induction of antibodies and clinical outcome. One reason for this failure might be the short duration of monitoring the patients. Hoon et al. [34] used an ELISA to monitor patients for anti-MAGE 1 IgG antibody and showed that peak titers were usually obtained 12–16 weeks after treatment, and such reactivity, if present, would be seen by that time. The other reason shall be a small scale of this study. Further studies are needed to elucidate this point.

DTH is a classical and not a quantitative test, but it is an effective method in some clinical studies. A resent study of stage III melanoma patients found that repeated immunization with a polyvalent, allogeneic melanoma cell vaccine induced a DTH response that correlated with overall survival [35]. Interestingly, the present study showed that four of six patients who demonstrated a positive skin test for DTH reactions experienced a reduction in PSA levels after vaccinations, whereas only one patient who remained skin test negative demonstrated a reduction in PSA.

In conclusion, this clinical trial demonstrated CTL precursor-oriented peptide vaccine is feasible, safe, and immunologically active. Clinical activity appears to be present, although confirmation of a clinical benefit will require completion of further trials. This trial may establish the groundwork for future refinements, including the use in patients with less extensive disease and a possibility of combination with other therapeutic agents or modalities.

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