# Prostate-Specific Antigen-Derived Epitopes Capable of Inducing Cellular and Humoral Responses in HLA-A24$^+$ Prostate Cancer Patients

Mamoru Harada,[1]* Kazuhiko Kobayashi,[1] Satoko Matsueda,[1] Masami Nakagawa,[1] Masanori Noguchi,[2] and Kyogo Itoh[1]

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

**BACKGROUND.** We tried to identify prostate-specific antigen (PSA)-derived epitopes immunogenic in HLA-A24$^+$ prostate cancer patients.
**METHODS.** Peripheral blood mononuclear cells (PBMCs) were in vitro stimulated with each of four different PSA peptides carrying the HLA-A24 binding motif, and their HLA-A24-restricted anti-tumor responses were examined using a parental HLA-A24-negative prostate cancer cell line (PC93) and its HLA-A24-expressing transfectant line (PC93-A24). Serum levels of immunoglobulin G (IgG) against PSA peptides were measured by enzyme-linked immunosorbent assay (ELISA).
**RESULTS.** PBMCs, which were in vitro stimulated with either the $PSA_{152-160}$ or $PSA_{248-257}$ peptide, showed higher levels of IFN-γ production and cytotoxicity against the PC93-A24 than against the PC93. IgG against the $PSA_{248-257}$ peptide was detected in half of the prostate cancer patients tested.
**CONCLUSIONS.** The $PSA_{152-160}$ and $PSA_{248-257}$ peptides could be appropriate target molecules in use for specific immunotherapy of HLA-A24$^+$ prostate cancer patients. *Prostate 57: 152–159, 2003.* © 2003 Wiley-Liss, Inc.

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

## INTRODUCTION

Prostate cancer is the most common malignancy in elderly men [1]. Hormone therapy has been therapeutically applied for patients with metastatic prostate cancer, although advanced prostate cancer becomes refractory to hormonal manipulation. Therefore, new treatment modalities are required. One such candidate treatment is anti-cancer immunotherapy. Recent advances in tumor immunology have enabled the identification of many genes encoding tumor antigens and peptides recognized by cytotoxic T lymphocytes (CTLs) [2–7]. Vaccination with melanoma antigen-derived peptides has been applied to elicit melanoma-reactive CTLs in patients, and clinical responses have been reported in some melanoma patients [8–10]. In addition, immunotherapy with antibodies, including anti-HER2/neu and anti-EGF receptor monoclonal

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

Grant sponsor: Ministry of Education, Science, Sports, and Culture of Japan; Grant sponsor: Tokyo Biomedical Research Foundation of Japan; Grant sponsor: Fukuoka Cancer Society.

*Correspondence to: Mamoru Harada, MD, PhD, Department of Immunology, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan.
E-mail: haramamo@med.kurume-u.ac.jp
Received 3 September 2002; Accepted 26 February 2003
DOI 10.1002/pros.10280
Published online 22 August 2003 in Wiley InterScience (www.interscience.wiley.com).

antibodies (mAbs), was reported to eliminate certain malignant diseases [11,12].

Prostate cancer cells express tissue-specific antigens, including prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and prostatic acid phosphatase (PAP). Several epitopic peptides of these antigens capable of inducing tumor-reactive CTLs were identified [13–15], and efficacy of immunotherapy with PSMA peptides in combination with dendritic cells has been reported [16–19]. However, information about immunogenic epitopes of either PSA or PSMA has been limited to that concerning HLA-A2$^+$ patients. In order to extend the application of PSA-targeting immunotherapy for prostate cancer patients, in this study, we attempted to identify PSA-derived epitopes immunogenic in HLA-A24$^+$ prostate cancer patients.

## MATERIALS AND METHODS

### Patients

All of the HLA-A24$^+$ prostate cancer patients and HLA-A24$^+$ healthy volunteers in this study were enrolled after informed consent was obtained. 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 and healthy donors was determined by flow cytometry, as previously reported [6].

### Cell Lines

C1R-A2402 is an HLA-A2402-expressing subline of C1R lymphoma, continuously cultured in RPMI-1640 medium supplemented with 10% FCS and 500 μg/ml hygromycin B (Gibco-BRL, Grand Island, NY). PC93 is an HLA-A24-negative prostate cancer cell line that was established by Dr. K. Ohishi (Department of Urology, Kyoto University, Japan), and PC93-A24 is a subline that was stably transfected with the *HLA-A2402* gene. The expression of HLA-A24 molecules on PC93-A24 was previously reported [20].

### Peptides

Four PSA-derived peptides were prepared based on the HLA-A24 binding motif [21,22] and they are listed in Table I. All peptides were of >90% purity and were purchased from Biologica Co. (Nagoya, Japan). Influenza (Flu) virus-derived, Epstein-Barr virus (EBV)-derived, and HIV-derived peptides with the HLA-A24 binding motif were used as controls. All peptides were dissolved with dimethyl sulfoxide at a dose of 10 mg/ml.

### Detection of Peptide-Specific CTL Precursors

A method to detect peptide-specific CTL precursors in PBMCs was previously reported [23]. In brief, PBMCs ($1 \times 10^5$ cells/well) were incubated with 10 μg/ml of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) at a

**TABLE I. The Tested PSA Peptides and Reactivity of PSA Peptide-Stimulated PBMCs From HLA-A24$^+$ Healthy Donors**

| | | | Healthy donors | | | | |
|---|---|---|---|---|---|---|---|
| Peptide | Sequence | Score[a] | #1 | #2 | #3 | #4 | #5 |
| | | | IFN-γ production (pg/ml)[b] | | | | |
| $PSA_{1-10}$ | MWVPVVFLTL | 12.1 | 30 | 0 | 0 | 7 | 0 |
| $PSA_{2-10}$ | WVPVVFLTL | 10.1 | 14 | 0 | 0 | 0 | 0 |
| $PSA_{152-160}$ | CYASGWGSI | 50.0 | 666 | 0 | 0 | 3 | 0 |
| $PSA_{248-257}$ | HYRKWIKDTI | 70.0 | 0 | 156 | 0 | 0 | 0 |
| Flu | RFYIQMCYEL | | 767 | 197 | 152 | 0 | 177 |
| EBV | TYGPVFMCL | | 277 | 248 | 48 | 81 | 120 |

PSA, prostate-specific antigen; PBMCs, peripheral blood mononuclear cells; Flu, influenza; EBV, Epstein-Barr virus.

[a]Score represents an estimated half-time of dissociation of the PSA peptide binding to HLA-A24 molecules.

[b]PBMCs of HLA-A24$^+$ healthy donors were in vitro stimulated with the indicated PSA peptide, as described in Materials and Methods. On day 12, the cultured PBMCs were tested for their reactivity to a corresponding peptide. Values represent the means of IFN-γ production by the effector PBMCs in response to C1R-A24 cells pulsed with a corresponding peptide. Background IFN-γ in response to HIV peptide (<150 pg/ml) was subtracted. Significant values ($P < 0.05$ by two-tailed Student's *t*-test) are underlined.

volume of 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 non-essential 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 12th day of culture, the harvested cells were tested for their ability to produce interferon (IFN)-γ in response to C1R-A2402 cells ($1 \times 10^4$ cells/well), which were pre-loaded with either a corresponding peptide or the HIV peptide as a negative control. After an 18-hr incubation, the supernatant (100 μl) was collected, and the level of IFN-γ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 16 pg/ml).

### Cytotoxicity Assay

The peptide-stimulated PBMCs were further cultured in order to obtain a sufficient number of cells to carry out a cytotoxicity assay, as reported previously [5]. These cells were tested for cytotoxicity against both PC93 and PC93-A24 by a 6-hr $^{51}$Cr-release assay [4].

### Detection of Peptide-Specific Immunoglobulin G (IgG)

Peptide-specific IgG levels in sera were measured by ELISA, as previously reported [24]. Briefly, a peptide (20 μg/well)-immobilized plates were blocked with Block Ace (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween-20–PBS. One hundred microliters/well of plasma sample diluted with 0.05% Tween-20-Block Ace was added to each plate. After a 2-hr incubation at 37°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 plates were washed, and then 100 μl of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase (En Vision, DAKO) was added to each well. The plates were then incubated at room temperature for 40 min. After another washing, 100 μl/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of 1 M phosphoric acid. To estimate peptide-specific IgG levels, we compared the optical density (OD) values of each sample with those of serially diluted standard samples; these values are shown as OD U/ml. To confirm the specificity of IgG to the $PSA_{248-357}$ peptide, 100 μl of sample serum was cultured in plates coated with either the $PSA_{248-257}$ peptide or the $PSA_{152-160}$ peptide. Thereafter, the level of $PSA_{248-257}$ peptide-specific IgG in the resultant supernatant was determined by ELISA.

### Statistics

The statistical significance of the data was determined using two-tailed Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

## RESULTS

### Induction of Prostate Cancer-Reactive CTLs by PSA Peptides From Healthy Donors

PBMCs of five HLA-A24$^+$ healthy donors were incubated with each of four kinds of PSA-derived peptide or with Flu and EBV peptides as controls (Table I). The $PSA_{1-10}$ and $PSA_{2-10}$ peptides failed to induce peptide-specific CTLs from any PBMCs of five healthy donors. The $PSA_{152-160}$ and $PSA_{248-257}$ peptides induced peptide-specific CTLs in one of five healthy donors. Flu peptide-specific and EBV peptide-specific CTLs were induced in four of five healthy donors. To determine whether these two PSA peptides could induce prostate cancer-reactive CTLs, the PBMCs of healthy donors #1 and #2 were newly stimulated with either the $PSA_{152-160}$ or the $PSA_{248-257}$ peptide and were examined for their IFN-γ production in response to prostate cancer cells. As a result, the $PSA_{152-160}$ peptide-stimulated and the $PSA_{248-257}$ peptide-stimulated PBMCs from healthy donors #1 and #2, respectively, produced higher levels of IFN-γ against the PC93-A24 cells than against the PC93 cells (Fig. 1A). Thereafter, the remaining peptide-stimulated PBMCs were additionally cultured for 14 days with only IL-2, and were examined for their cytotoxicity. These cultured cells exhibited higher cytotoxic activity against the PC93-A24 cells than against the PC93 cells (Fig. 1B). These results indicate that these two PSA peptides possess the immunogenicity to induce HLA-A24-restricted and prostate cancer-reactive CTLs from the PBMCs of HLA-A24$^+$ healthy donors.

### Induction of Prostate Cancer-Reactive CTLs by PSA Peptides From Cancer Patients

PBMCs from 20 HLA-A24$^+$ prostate cancer patients were further tested, and nine cases in which peptide-specific CTLs were induced are shown in Table II. The data of patient #9 was not included in this table because frozen PBMCs from this patient were not recovered. Both the $PSA_{1-10}$ and $PSA_{2-10}$ peptide induced peptide-specific CTLs in only one patient. The $PSA_{152-160}$ and $PSA_{248-257}$ peptides could induce peptide-specific CTLs in five and eight out of 20 patients, respectively. Based on these findings, the $PSA_{152-160}$ and $PSA_{248-257}$ peptides were the focus of the following experiments.

It was further determined whether PBMCs stimulated by either the $PSA_{152-160}$ or the $PSA_{248-257}$ peptide

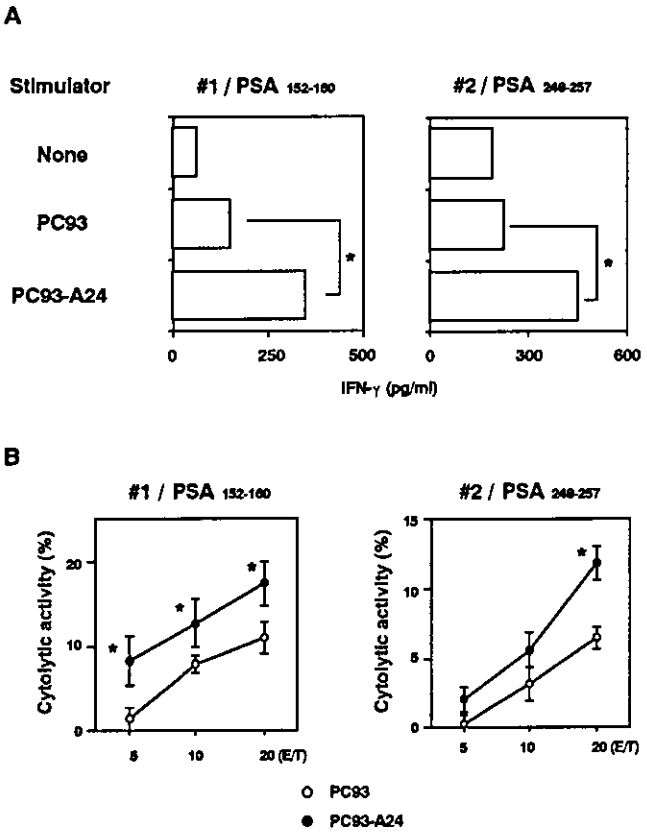


**Fig. 1.** HLA-A24-restricted responses of the prostate-specific antigen (PSA) peptide-stimulated peripheral blood mononuclear cells (PBMCs) from healthy donors. **A**: PBMCs from two healthy donors were cultured with either the $PSA_{152-160}$ or the $PSA_{248-257}$ peptide, as described in Materials and Methods. On day 12, the cultured cells were examined for their IFN-γ production against the PC93 cells and PC93-A24 cells. $^*P < 0.05$ was considered statistically significant. **B**: The remaining cells were cultured for additional 14 days, and were examined for their cytotoxicity against the PC93 cells and PC93-A24 cells by a 6-hr $^{51}Cr$-release assay. Values represent the mean of triplicate assays. $^*P < 0.05$ was considered statistically significant.

could induce prostate cancer-reactive CTLs from HLA-A24$^+$ prostate cancer patients. For this assay, PBMCs from four cancer patients (#1, #8, and #10 in Table II and another patient, #9) were stimulated with either of these two PSA peptides and were examined for their IFN-γ production in response to prostate cancer cells (Fig. 2A). PBMCs were again obtained from the patient #9 to carry out this experiment. As a consequence, the $PSA_{152-160}$ peptide induced prostate cancer-reactive CTLs from patients #8 and #10, whereas the $PSA_{248-257}$ peptide induced prostate cancer-reactive CTLs from patients #1, #8, and #10. After additional culture with IL-2 alone for 2 weeks, these peptide-stimulated PBMCs were examined for their cytotoxicity against prostate cancer cells (Fig. 2B). These PSA peptide-stimulated PBMCs showed higher levels of

**TABLE II. Representative Results of PSA Peptide-Stimulated PBMCs From HLA-A24$^+$ Patients With Prostate Cancer**

| | Prostate cancer patient | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Peptide | #1 | #2 | #3 | #4 | #5 | #6 | #7 | #8 | #10 |
| | IFN-γ production (pg/ml)[a] | | | | | | | | |
| $PSA_{1-10}$ | 178 | 16 | 5 | 22 | 4 | 6 | 33 | ND | ND |
| $PSA_{2-10}$ | 48 | 25 | 0 | 18 | 125 | 0 | 0 | ND | ND |
| $PSA_{152-160}$ | 390 | 6 | 207 | 9 | 36 | 183 | 32 | 84 | 80 |
| $PSA_{248-257}$ | 224 | 89 | 169 | 90 | 232 | 0 | 170 | 150 | 69 |
| Flu | 122 | 0 | 84 | 31 | 0 | 0 | 2 | ND | ND |
| EBV | 225 | 270 | 61 | 279 | 0 | 20 | 189 | 123 | 108 |

[a]PBMCs of HLA-A24$^+$ prostate cancer patients were tested for their reactivity to a corresponding peptide after incubation with each peptide for 12 days. Representative nine results of 20 prostate cancer patients are shown. Values represent the means of IFN-γ production by the effector PBMCs in response to C1R-A24 cells pulsed with a corresponding peptide. Background IFN-γ in response to HIV peptide (<150 pg/ml) was subtracted. Significant values ($P < 0.05$ by two-tailed Student's *t*-test) are underlined. ND; not done.

cytotoxicity against the PC93-A24 cells than against the PC93 cells. The $PSA_{152-160}$ peptide-stimulated PBMCs from patient #1 were not expanded for CTL assay because the difference in IFN-γ production in response to PC93 versus PC93-A24 was not significant, as shown in Figure 2A. In general, these results indicate that these $PSA_{152-160}$ and $PSA_{248-257}$ peptides have the capacity of inducing HLA-A24-restricted and prostate cancer-reactive CTLs from HLA-A24$^+$ prostate cancer patients.

### Detection of IgG-Specific to the $PSA_{248-257}$ Peptide

Sera from ten HLA-A24$^+$ prostate cancer patients with the median age of 67.8 (range 50–86) were further investigated in order to determine whether or not IgG-specific to any of the four PSA peptides could be identified. Sera in five of ten cancer patients (50%) did contain IgG-specific to the $PSA_{248-257}$ peptide, as shown in Figure 3A. No IgG reactive to the other three PSA peptides was detected in the sera of ten patients, with the one exception that a relatively low level of IgG against the $PSA_{152-160}$ peptide was detected in serum of patient #11. The specificity of IgG against the $PSA_{248-257}$ peptide was then confirmed by an experiment in which anti-$PSA_{248-257}$ peptide IgG in the serum of patient #1 was significantly absorbed by incubation with the $PSA_{248-257}$ peptide-coated plate, but not with the $PSA_{152-160}$ peptide-coated plate (Fig. 3B). In addition, sera of ten HLA-A24$^+$ healthy donors were examined to determine whether or not IgG-specific to these two PSA peptides was present. Ten healthy

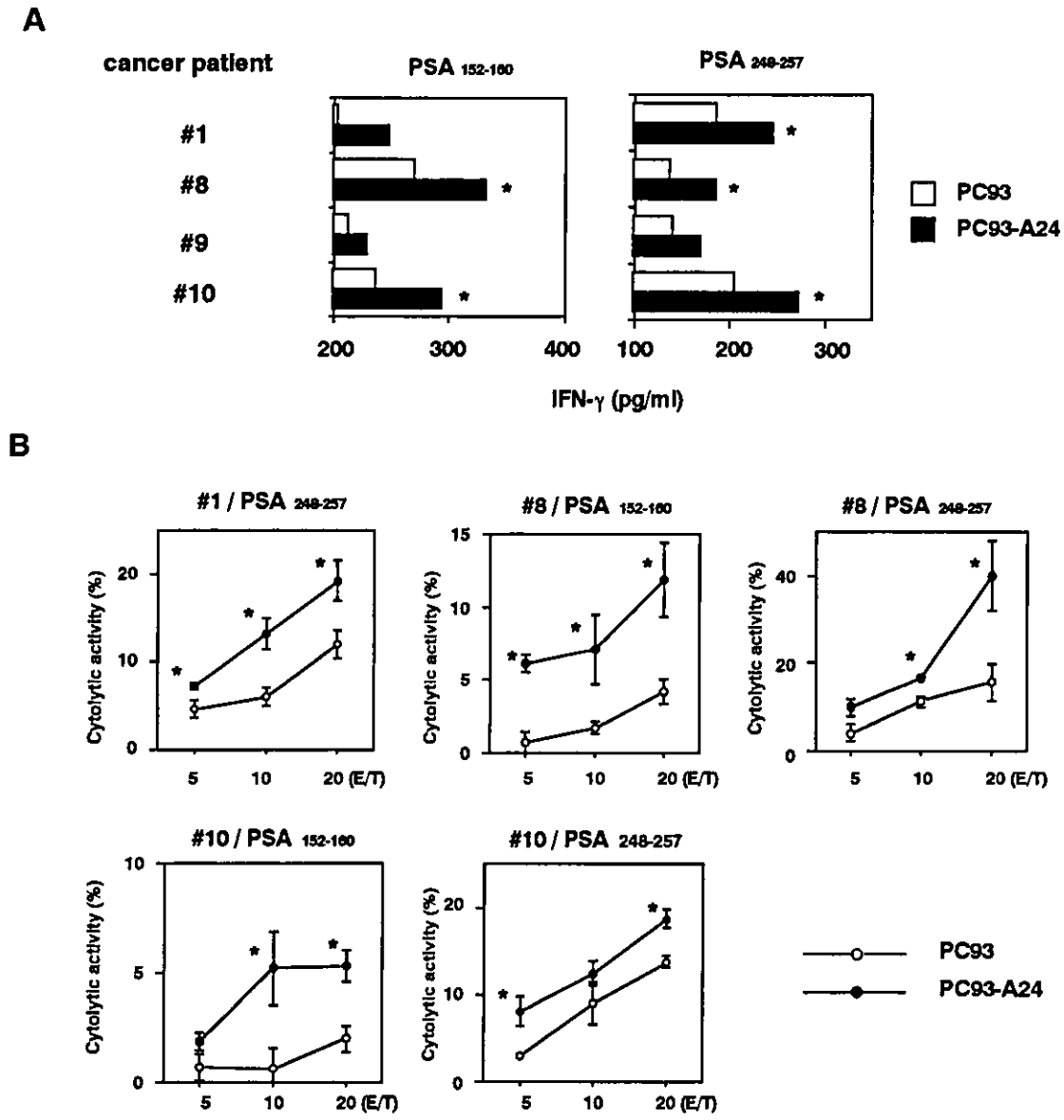


**Fig. 2.** HLA-A24-restricted responses of the PSA peptide-induced PBMCs-derived from cancer patients. **A**: PBMCs from four HLA-A24$^+$ cancer patients were cultured with either the $PSA_{152-160}$ or the $PSA_{248-257}$ peptide, as described in Materials and Methods. On day 12, the cultured cells were examined for their IFN-γ production against the PC93 cells and PC93-A24 cells. *$P < 0.05$ was considered statistically significant. **B**: The remaining cells were cultured for additional 14 days, and were examined for their cytotoxicity against the PC93 cells and the PC93-A24 cells by a 6-hr $^{51}$Cr-release assay. Values represent the mean of triplicate assays. *$P < 0.05$ was considered statistically significant.

donors consist of seven males and three females, and their median ages were 31.6 (range 24–37) and 33.7 (range 29–43), respectively. It was found that sera from eight (five males and three females) healthy donors contained IgG reactive to the $PSA_{248-257}$ peptide (Fig. 3C).

## DISCUSSION

PSA is a kallikrein-like serine protease, and human PSA has a high degree of homology with human pancreatic kallikrein [25]. Immunoperoxidase staining indicates that PSA is found in the cytoplasmic portion of these cells. In addition, PSA is well-known as a tumor marker in prostate cancer patients [26]. For these reasons, PSA is a potential target for immunotherapy against prostate cancer. We showed in this study that PBMCs from HLA-A24$^+$ patients with prostate cancer showed a peptide-specific IFN-γ production in five of 20 patients (25%) and eight of 20 patients (40%) when stimulated with the $PSA_{152-160}$ and the $PSA_{248-257}$ peptide, respectively. In addition, these PSA peptide-recognizing CTLs were found to show reactivity against prostate cancer cells in an HLA-A24-restricted manner. These lines of evidence indicate that these

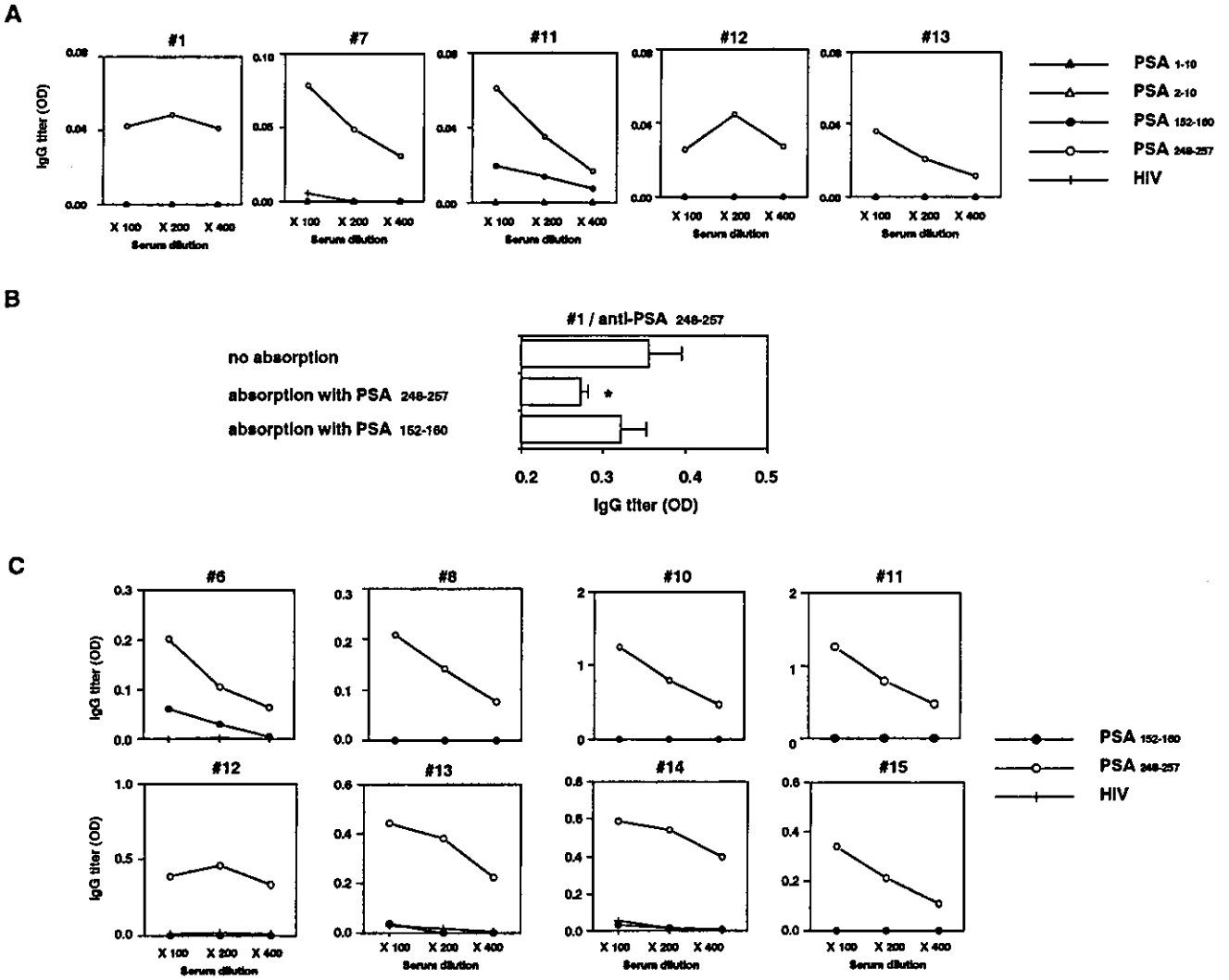


**Fig. 3.** Detection of IgG reactive to the $PSA_{248-257}$ peptide in sera of both prostate cancer patients and healthy donors. **A**: Five positive results of cancer patients are shown. These values are shown as optical density (OD) U/ml. The HIV peptide was used as a negative control. **B**: To confirm the specificity of IgG to the $PSA_{248-257}$ peptide, 100 μl of sample serum from patient #1 was cultured in a plate coated with either the $PSA_{248-257}$ peptide or the $PSA_{152-160}$ peptide. Thereafter, the level of IgG reactive to the $PSA_{248-257}$ peptide in the resultant sample was determined. **C**: Eight positive results of healthy donors are shown. The levels of IgG reactive to the $PSA_{152-160}$ and $PSA_{248-257}$ peptides were determined. The HIV peptide was used as a negative control.

two PSA peptides are potent peptides for generating tumor-reactive CTLs in HLA-$A24^+$ patients with prostate cancer.

The $PSA_{152-160}$ and $PSA_{248-257}$ peptides induced peptide-specific CTLs in PBMCs of certain HLA-$A24^+$ healthy donors. In addition, PBMCs in vitro stimulated with either the $PSA_{152-160}$ or the $PSA_{248-157}$ peptide demonstrated cytotoxicity against prostate cancer cells in an HLA-A24-restricted manner. This result is consistent with a previous report that CTLs reactive to PSA can be induced from PBMCs of HLA-$A2^+$ healthy donors [27]. In addition, we also previously reported that PAP-derived peptide-reactive CTLs could be induced from PBMCs of HLA-$A24^+$ healthy donors by in vitro stimulation with PAP peptides [20]. A similar phenomenon was observed with regard to melanocyte differentiation antigens. That is, melanocyte differentiation antigen-reactive CTLs can be induced from healthy donors [28]. When taken together, these results suggest that CTL precursors reactive to non-mutated self antigens circulate in the peripheral blood of both certain healthy donors and cancer patients.

The immunogenicity of the $PSA_{248-257}$ peptide appeared to be superior to that of the $PSA_{152-160}$ peptide. One explanation is that this was due to the difference in their binding scores, i.e., an estimated half-time dissociation binding to the HLA-A24 molecules, as shown in Table I. In addition, negative

CTL responses were observed, especially with the $PSA_{152-160}$ peptide. We suppose that such observations may have been cased either by insufficient CTL induction by PSA peptides or by peptide modification. The first position of the $PSA_{152-160}$ peptide has cysteine, which can be easily oxidized as a result of disulfide bondage. MHC class-I binding peptides containing cysteine have been reported to have decreased immunogenicity for T-cell as a result of either cystinylation or dimerization [29]. Substitution of cysteine in the $PSA_{152-160}$ peptide with either alanine or serine has been shown to restore the immunogenicity, as previously reported [30].

We determined whether antibodies against PSA peptides were detectable in sera from HLA-$A24^+$ prostate cancer patients because antibodies against class I tumor peptides were detected in certain cancer patients [31,32]. As a result, IgG-specific to the $PSA_{248-257}$ peptide was detected in five of ten prostate cancer patients (50%). Furthermore, this IgG was detected in eight of ten healthy donors (80%). Both the positive percentage and the titer (OD) of IgG reactive to the $PSA_{248-257}$ peptide were higher in healthy donors than in prostate cancer patients. Because in vivo generation of antigen-specific IgG generally requires antigen-specific cytokine help from T-cell, detection of $PSA_{248-257}$ peptide-specific IgG might suggest an in vivo response of PSA-reactive helper T-cell. These findings may indicate that helper T-cell responses to PSA, a non-mutated self antigen, might deviate to the Th2-type in healthy donors. On the other hand, several researchers have reported successful immunotherapy of malignant diseases with mAbs recognizing non-mutated self-antigens, including Rituximab (ant-CD20 mAb) [33], Trastuzumab (anti-HER2) [12], and IRRESA (anti-EGF receptors) [11]. It remains of interest to clarify the roles played by IgG against self antigen-derived peptides in cancer patients and healthy donors.

In conclusion, we identified two epitopes of PSA immunogenic in HLA-$A24^+$ prostate cancer patients. The $PSA_{152-160}$ peptide was recently reported to be immunogenic in HLA-A2402/$K^b$ transgenic mice [34]. The frequencies of the HLA-A24 allele are relatively high in Japanese (60%), Caucasians (20%), Chinese (25%), Hispanics (30%), and North American black populations (7%) [35]. The present finding will hopefully contribute to the development of specific immunotherapy for HLA-$A24^+$ prostate cancer patients.

## ACKNOWLEDGMENTS

We thank Dr. K. Ohishi (Department of Urology, Kyoto University, Kyoto) and Dr. Y. Inoue (Pharmacology Division, National Cancer Center, Tokyo) for providing us with the PC93 and the PC93-A24 cell lines, respectively.

## REFERENCES

1. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7–33.
2. Boon T, Coulie PG, Van den Eynde B. Tumor antigens recognized by T cells. Immunol Today 1997;81:267–268.
3. Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 1999;10: 281–287.
4. Shichijo S, Nakao M, Imai Y, Takasu H, Kawamoto M, Niiya F, Yang D, Toh Y, Yamana H, Itoh K. A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. J Exp Med 1998;187:277–288.
5. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, Mori K, Kayashi A, Yamana H, Shirouzu H, Itoh K. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. Cancer Res 1999;59: 4056–4063.
6. Kawano K, Gomi S, Tanaka K, Tsuda N, Kamura T, Itoh K, Yamada A. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A24-restricted tumor infiltrating lymphocytes of lung cancer. Cancer Res 2000;60: 3550–3558.
7. Nakao M, Shichijo S, Imaizumi T, Inoue Y, Matsunaga K, Yamada A, Kikuchi M, Tsuda N, Ohta K, Takamori S, Yamana H, Fujita H, Itoh K. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. J Immunol 2000;164:2565–2574.
8. Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4: 328–332.
9. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudly ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321–327.
10. Marchand M, Van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Lienard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jager E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, van der Bruggen P, Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. Int J Cancer 1999;80:219–230.
11. Goldstein NI, Prewett M, Zuklys K, Rockwell P, Mendelsohn J. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. Clin Cancer Res 1995;1:1311–1318.
12. Burstein HJ, Kuter I, Campos SM, Gelman RS, Tribou L, Parker LM, Manola J, Younger J, Matulonis U, Bunnell CA, Partridge AH, Richardson PG, Clark K, Shulman LN, Winer EP. Clinical activity of trastuzumab and vinorelbine in woman with HER2-overexpressing metastatic breast cancer. J Clin Oncol 2001;19: 2722–2730.
13. Correale P, Walmsley K, Nieroda C, Zaremba S, Zhu M, Schlom J, Tsang KY. In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. J Natl Cancer Inst 1997;89:293–300.
14. Xue BH, Zhang Y, Sosman J, Peace DJ. Induction of human cytotoxic T lymphocytes specific for prostate-specific antigen. Prostate 1997;30:73–78.

15. Correale P, Walmsley K, Zaremba S, Zhu MZ, Schlom J, Tsang KY. Generation of human cytotoxic T lymphocyte lines directed against prostate-specific (PSA) employing a PSA oligoepitope peptide. J Immunol 1998;161:3186–3194.
16. Murphy GP, Tjoa BA, Ragde H, Kenny G, Boynton A. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. Prostate 1996;29:371–380.
17. Murphy GP, Tjoa BA, Simmons SJ, Jarisch J, Bowes VA, Ragde H, Rogers M, Elgamal A, Kenny GM, Cobb OE, Ireton RC, Troychak MJ, Salgaller ML, Boynton AL. Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: A phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease. Prostate 1999;38: 73–78.
18. Murphy GP, Tjoa BA, Simmons SJ, Ragde H, Rogers M, Elgamal A, Kenny GM, Troychak MJ, Salgaller ML, Boynton AL. Phase II prostate cancer vaccine trial: Report of a study involving 37 patients with disease recurrence following primary treatment. Prostate 1999;39:54–59.
19. Tjoa BA, Simmons SJ, Elgamal A, Rogers M, Ragde H, Kenny GM, Troychak MJ, Boynton AL, Murphy GP. Follow-up evaluation of a phase II prostate cancer vaccine trial. Prostate 1999;40:125–129.
20. Inoue Y, Takaue Y, Takei M, Kato K, Kanai S, Harada Y, Tobisu K, Noguchi M, Kakizoe T, Itoh K, Wakazugi H. Induction of tumor specific cytotoxic T lymphocytes in prostate cancer using prostatic acid phosphatase derived HLA-A2402 binding peptide. J Urol 2001;166:1508–1513.
21. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152: 163–175.
22. Rammensee HG, Friege T, Stevanovic S. MHC ligands and peptides motifs. Immunogenetics 1995;41:178–228.
23. Hida N, Maeda Y, Katagiri K, Takasu H, Harada M, Itoh K. A new culture protocol to detect peptide-specific cytotoxic T lymphocyte precursors in the circulation. Cancer Immunol Immunl 2002;51:219–228.
24. Ohkouchi S, Yamada A, Imai N, Mine T, Harada K, Shichijo S, Maeda Y, Saijo Y, Nukiwa T, Itoh K. Non-mutated tumor-rejection antigen peptides elicit type-I allergy in the majority of healthy individuals. Tissue Antigens 2002;59:259–272.
25. Lundwell A, Lilija H. Molecular cloning of human prostate specific antigen. FEBS Lett 1989;214:317–320.
26. Oesterling JE. Prostate specific antigen: A critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. J Urol 1991;145:907–923.
27. Heiser A, Dahm P, Yancey DR, Maurice MA, Boczkowski D, Nair SK, Gilboa E, Vieweg J. Human dendritic cells transfected with RNA encoding prostate-specific antigen stimulate prostate-specific CTL responses in vitro. J Immunol 2000;164:5508–5514.
28. Pittet MJ, Valmori D, Dunbar PR, Speiser DE, Lienard D, Lejeune F, Fleischhauer K, Cerundolo V, Cerottini JC, Romero P. High frequencies of naïve melan-A/MART-1-specific $CD8^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. J Exp Med 1999;190:705–716.
29. Meadows L, Wang W, den Haan JMM, Drijfhout JW, Shabanowitz J, Pierce R, Agulnik AI, Bishop CE, Hunt DF, Goulmy E, Engelhard VH. The HLA-A*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition. Immunity 1997;6:273–281.
30. Kittlesen D, Thompson LW, Gulden PH, Skiller JCA, Colella TA, Shabanowitz JA, Hunt DR, Engelhard VH, Slingluff JC. Human melanoma patients recognize an HLA-A1-restricted CTL epitope from tyrosinase containing two cysteine residues: Implications for tumor vaccine development. J Immunol 1998; 160:2099–2106.
31. Miyagi Y, Imai N, Sasatomi T, Yamada A, Mine T, Katagiri K, Nakagawa M, Muto A, Ohkouchi S, Isomoto H, Shirouzu K, Yamana H, Itoh K. Induction of cellular immune responses to tumor cells and peptides in colorectal cancer patients by vaccination with SART3 peptides. Clin Cancer Res 2001;7:3950–3962.
32. Nakatsura T, Senju S, Ito M, Nishimura Y, Itoh K. Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur J Immunol 2002;32:826–836.
33. Alas S, Bonavida B. Rituximab inactivates signal transducer and activation of transcription 3 (STAT 3) activity in B-non-Hodgkin's lymphoma through inhibition of the interleukin 10 autocrine/paracrine loop and results in down-regulation of bcl-2 and sensitization to cytotoxic drugs. Cancer Res 2001;61: 5137–5144.
34. Gotoh M, Takasu H, Karada K, Yamaoka T. Development of HLA-A2402/$K^b$ transgenic mice. Int J Cancer 2002;100:565–570.
35. Imanishi T, Akazawa T, Kimura A. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasazuki T, editors. HLA 1991. Vol. 1. Oxford, England: Oxford Scientific Publications; 1992. pp 1065–1220.

# Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients

Takashi Mine,[1,2,3] Rumi Gouhara,[3,4] Naoya Hida,[1] Nobue Imai,[1] Kouichi Azuma,[1] Touru Rikimaru,[4] Kazuko Katagiri,[1] Misa Nishikori,[1] Aki Sukehiro,[1] Masami Nakagawa,[1] Akira Yamada,[5] Hisamichi Aizawa,[4] Kazuo Shirouzu,[2] Kyogo Itoh[1] and Hideaki Yamana[2]

[1]Department of Immunology, [2]Department of Surgery, and [4]First Department of Internal Medicine, Kurume University School of Medicine and [5]Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, 67 Asahi-machi, Kurume 830-0011

(Received January 27, 2003/Revised March 20, 2003/Accepted April 9, 2003)

**Recent clinical trials of peptide vaccine for cancer patients have rarely resulted in tumor regression. One of the reasons for this failure could be an insufficient induction of anti-tumor responses in these regimens, in which peptide-specific memory cytotoxic T lymphocytes (CTLs) were not measured prior to vaccination. We investigated in this study whether pre-vaccination measurement of peptide-specific CTLs can provide any advantages in lung cancer patients receiving peptide vaccination with regard to safety and immunological responses. Ten patients with advanced lung cancer received vaccination with peptides under a regimen of CTL precursor-oriented vaccination, in which pre-vaccination peripheral blood mononuclear cells (PBMCs) were at first screened for reactivity *in vitro* to each of 14 peptides, followed by *in vivo* administration of only the reactive peptides. Profiles of the vaccinated peptides varied markedly among the 10 patients. This regimen was generally well-tolerated, although local skin reactions, diarrhea, and colitis were observed in 8, 2, and 1 patient, respectively. Increased CTL responses against the immunized peptides and tumor cells were observed in the post-vaccination PBMCs from 4 of 8 and 3 of 10 patients tested, respectively. Peptide-specific IgG became detectable in post-vaccination sera in 4 of 10 patients tested, and these 4 patients had a long progression-free survival. Furthermore, the median survival time of 9 patients with non-small cell lung cancer was 668.0±164.2 days. These results encourage further development of CTL precursor-oriented peptide vaccination for lung cancer patients. (Cancer Sci 2003; 94: 548–556)**

Identification of a large number of antigenic epitopes[1–9] recognized by cytotoxic T lymphocytes (CTLs) reacting to tumor cells has opened the door to clinical trials of peptide-based immunotherapy for cancer patients.[10–16] Many clinical trials of peptide-based immunotherapy for malignant melanoma and other epithelial tumors have been conducted in the past decade, but major clinical responses were rarely obtained in these clinical studies, including our trial of a cyclophilin B (CypB) peptide vaccination for advanced lung cancer patients[17] and a SART3 peptide vaccination for advanced colorectal cancer patients.[18] One reason for this failure to obtain tumor regression could be an insufficient induction of anti-tumor responses in these vaccine regimens, in which peptide-specific memory T cells were not measured in pre-vaccination peripheral blood mononuclear cells (PBMCs). We have speculated that vaccination based on information from pre-vaccination measurement of peptide-specific CTLs in the circulation might induce potent anti-tumor immune responses in cancer patients. This hypothesis is based on the assumption that initiation of immune-boosting of CTLs through peptide vaccination could be more effective than immune-priming of naive T cells with regard to induction of prompt and strong immunity to both the peptide and tumor cells. We have recently reported a method of measuring peptide-specific CTLs in pre-vaccination PBMCs of cancer patients.[19,20] With this new method, we have measured peptide-specific CTL precursors in pre-vaccination PBMCs of lung cancer patients, and then vaccinated only the CTL-directed peptides, as a part of a phase I clinical study of CTL precursor-oriented vaccines. In this report, we describe the safety and immune responses to peptides and tumor cells in HLA-A24$^+$ advanced lung cancer patients vaccinated with these CTL-directed peptides.

## Patients and Methods

**Trial eligibility.** The institutional ethical review boards approved the study protocol (#2031). Full written informed consent was obtained from all patients at the time of enrollment. According to the protocol, patients were required to be HLA-A24 positive, to have a histologically confirmed lesion of either non-small cell or small cell lung cancer, to have been untreated for at least 4 weeks before the study, and to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2. Eligibility criteria included age from 20 to 84 years, a creatinine level less than 1.4 mg/dl, a bilirubin level less than 1.5 mg/dl, platelet count of more than 100 000/mm$^3$, hemoglobin of more than 8.0 g/dl, and total WBC of more 3000/mm$^3$. Hepatitis B and C antigens were required to be negative. The patients were also required to exhibit an absence of active infection, clinical heart failure, respiratory failure, renal failure, immunodeficiency, coagulopathy, and synchronous cancer. This clinical study was carried out at the Kurume University Hospital from November 2000 through April 2002.

**Peptides and selection for vaccination.** The peptides utilized in the present study were prepared under conditions of Good Manufacturing Practice using the Multiple Peptide System, San Diego, CA. The peptide sequences are shown in Table 2. All of these peptides have the ability to induce HLA-A24-restricted and tumor-specific CTL activity in PBMCs of cancer patients.[21–27] These peptides were dissolved and stored at −80°C. Stock solutions were diluted with saline just before use.

For the peptide screening, pre-vaccination PBMCs were provided for assays of peptide-specific CTL precursors by methods reported previously.[19] Pre-vaccination PBMCs were isolated from 30 ml of peripheral blood and incubated with 10 m*M* peptide in wells of U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark) at a concentration of $1\times10^5$ cells/well in 200 $\mu$l of culture medium. The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (Life Technologies, Gaithersburg, MD), 10% FCS, 100 U/ml of interleukin-2 (IL-2), and 0.1 m*M* MEM nonessential amino acid solution (Life Technologies). Half of the medium was removed and replaced with fresh medium containing a corresponding peptide (20 $\mu M$) every 3 days for up to 12 days. Twenty-four hours after the last stimulation, the cells were harvested and

[3]Two authors contributed equally to this work. E-mail: mine@med.kurume-u.ac.jp

tested for their ability to produce IFN-γ in response to CIR-A2402 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLLGI) as a negative control, by means of an enzyme-linked immuno-sorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). All experiments were performed in quadruplicate assays, and a two-tailed Student's *t* test was employed for the statistical analyses. Positive wells were evaluated in the following order: Criteria Ar, the peptide-specific IFN-γ production was ≥500 pg/ml and *P* value was <0.10; criteria A, the production was ≥50 pg/ml and *P* value<0.05; criteria B, 25≤ the production<50 pg/ml and *P* value<0.05; criteria C, the production was ≥50 pg/ml and 0.05≤*P* value<0.10; criteria D, the production was >100 pg/dl and 0.10≤*P* value<0.20. The peptide-specific IFN-γ production was calculated by subtraction of IFN-γ production of the peptide-stimulated PBMCs in response to a negative control (HIV peptide) from that in response to a corresponding peptide. According to the test results, up to four positive peptides were selected for each patient, and these were then vaccinated as CTL precursor-oriented peptide vaccine, if an immediate-type hypersensitivity reaction against each peptide was not seen in a skin test before vaccination. The screening of peptide-specific CTL precursors was also performed by the same method after the 6th and 12th vaccinations to evaluate the *in vivo* effects of vaccination.

**Clinical protocol.** Skin tests were performed by intradermal injection of 10 μg of each peptide using a tuberculin syringe and a 26-gauge needle. Saline was used as a negative control for assessment of hypersensitivity. Immediate- and delayed-type hypersensitivity (DTH) reactions were determined at 20 min and 24 h after the skin test, respectively. At least 5 mm of induration or 10 mm of erythema read 24 h after injection was needed to score the skin test as positive. If immediate-type hypersensitivity was negative, the peptide was vaccinated into the subcutaneous tissue of the upper arm or anterior thigh of patients. Two milliliters of the peptide, which was supplied in vials containing 2 mg/ml sterile solution, was mixed with an equal volume of incomplete Freund's adjuvant (IFA) (Montanide ISA-51; Seppic, Paris, France) and emulsified in 5-ml sterilized syringes. Each 3 ml of the preparing peptide (maximum 4 peptides at one vaccination) were injected three times every 2 weeks. Toxicity was assessed at least every 2 weeks using the National Cancer Institute's Common Toxicity Criteria (NCI-CTC). For patients with a favorable clinical course, the vaccinations were continued every 2 weeks with consent from each patient.

**$^{51}$Cr-release assay.** Cytotoxic activity was measured using a standard $^{51}$Cr-release assay, as reported previously.[18,24] In brief, cryopreserved PBMCs were thawed and cultured in the medium consisting of 100 U/ml of IL-2 alone for 21 days without peptide, followed by harvesting of cells for the assay. To avoid the bias of bioassays, PBMCs harvested at different times from a single patient were thawed at the same time and were used for the study. HLA-A24$^+$ 11-18 lung adenocarcinoma cells, HLA-A24$^-$ QG56 lung squamous cell carcinoma cells, and HLA-A24$^+$ PHA blasts, as normal cells, were used as target cells ($1\times10^3$/well).

**Detection of serum IgG levels.** An ELISA was used to detect the serum IgG levels specific to the administered peptides, as reported previously.[18] Briefly, 100 μl/well of serum samples diluted with 0.05% Tween 20-Block Ace (Yukijirushi, Tokyo) was added to the peptide (20 μg/well)-immobilized plate, after which the plate was blocked with Block Ace, and washed. After a 2-h incubation, the plate was washed and further incubated for 2 h with a 1:1000-dilluted rabbit anti-human IgG (DAKO, Glostrup, Denmark). The plate was washed, 100 μl of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase-dextran polymer (EnVision, DAKO) was then added to each well, and the plate was incubated for 40 min. After washing, 100 μl/well of tetramethylbenzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of 1 *M* phosphoric acid. 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.

**Evaluation of clinical responses.** Before and after the 3rd, 6th, and 9th vaccinations, all known sites of disease were evaluated by CT-scan or X-ray examination. Patients were assigned a response category according to RESPONSE EVALUTION CRITERIA IN SOLID TUMORS (RECIST criteria), revised version of the WHO criteria published in the WHO Handbook for reporting results of cancer treatment (Geneva, 1979) June 1999 (Final).

## Results

**CTL precursor cells in pre-vaccination PBMCs of the patients who received CypB vaccination.** Prior to initiation of CTL precursor-oriented vaccination, peptide-specific CTL precursors in pre-vaccination PBMCs from the 6 lung cancer patients who had received $CypB_{91}$-peptide vaccination during 1999–2000 were detected retrospectively to confirm that peptide-specific CTL precursor cells varied among these patients. One to four kinds of peptide-specific CTL precursor cells were detected in all 6 patients. CTL precursors reactive to $SART2_{93}$, $SART2_{161}$, $SART3_{109}$, $CypB_{84}$, $CypB_{91}$, $ART4_{13}$, or $ART4_{75}$ peptide were detectable in pre-vaccination PBMCs of 1, 1, 4, 1, 3, 1, or 2 patients, respectively (Fig. 1A). These results indicate that pre-vaccination PBMCs of lung cancer patients possess detectable levels of peptide-specific CTL precursor cells, the profile of positive peptides varied among patients, and only 3 patients had CTL precursors reactive to the $CypB_{91}$ peptide that was vaccinated *in vivo*.[17]

**Patient population.** Ten patients with advanced lung cancer (their demographics are shown in Table 1) were enrolled in this CTL precursor-oriented vaccination regimen. The median age of patients was 64.9 years (range; 59–74). The histological diagnosis was adenocarcinoma ($n$=8), squamous cell carcinoma ($n$=1) and small cell carcinoma ($n$=1). Patients' performance status evaluated by ECOG criteria was 0 ($n$=7), 1 ($n$=2) or 2 ($n$=1). The therapeutic histories were post-operative recurrence ($n$=6), failure to respond to chemotherapy ($n$=5), and radiotherapy ($n$=3). No patient had any concurrent treatments, steroids, or any other immunosuppressive drugs for 4 weeks prior to the vaccination. The clinical stages of the patients were IV ($n$=3), and IIIb ($n$=1) of the UICC-cTNM classification, and recurrent cases ($n$=6) after surgical operation.

**Pre-vaccination measurement of peptide-specific CTL precursors.** Pre-vaccination PBMCs of each patient were provided for peptide screening. Representative results of two cases (EBL-005 and -006) are shown in Fig. 1B. One of the four wells of pre-vaccination PBMCs of 1 patient (EBL-005) responded to the $SART1_{690}$, $SART2_{93}$, $SART2_{161}$, and $SART3_{315}$ peptide with criteria of D, A, A, and C, respectively. In contrast, pre-vaccination PBMCs of the other patient (EBL-006) responded to the $SART1_{690}$ (the criteria of D), $SART3_{315}$ (Ar, A, and C), $CypB_{91}$ (D), and $ART1_{170}$ (C), respectively. Overall, CTL precursors reacting to 10, 5, 4, or 3 peptides were detected in pre-vaccination PBMCs of 1, 4, 4, or 1 patient, respectively (Table 2). Neither $CypB_{84}$ nor $ART1_{170}$ was vaccinated due to immediate-type hypersensitivity at the pre-vaccination skin-test. Subsequently, 4 or 3 peptides were injected in 7 or 3 patients, respectively. It is of note that the profile of vaccinated peptides greatly varied among these 10 patients. The frequencies of vaccinated peptides was as follows: $SART3_{315}$ for 5 of 10 patients; $SART1_{690}$, $SART2_{93}$, $SART2_{161}$, $lck_{208}$, and $lck_{488}$ for 4 patients; $SART2_{899}$ and $SART3_{109}$ for 3 patients; $CypB_{91}$ and $lck_{486}$ for 2

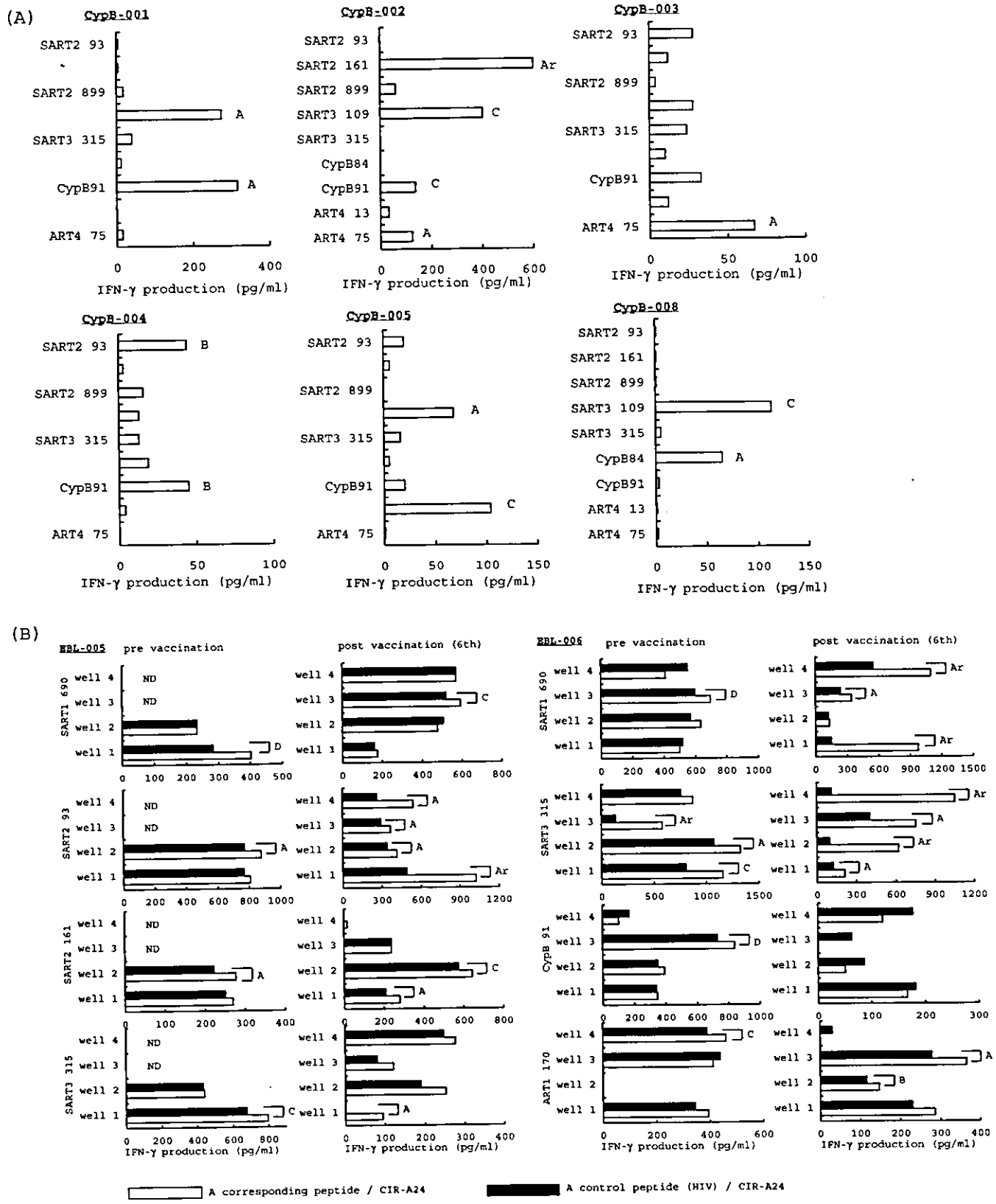


**Fig. 1.** Peptide-specific CTL precursors. (A) CTL precursors in pre-vaccination PBMCs from patients receiving a $CypB_{91}$ vaccination during 1999–2000. Values represent the amounts of IFN-γ produced by PBMCs in response to a corresponding peptide. IFN-γ production in response to a control peptide (HIV) was subtracted in the figure. Criteria for evaluation were described in "Materials and Methods." $CypB_{91}$-specific CTL precursors were detected in 3 patients (CypB-001, -002, and -004). (B) Peptide-specific CTL precursors were measured in pre- and post (6th)-vaccination PBMCs of all 10 and 7 patients, respectively. Representative results of IFN-γ production of EBL-005 and -006 are shown in this figure. A summary is provided in Table 4.

**Table 1. Patients' characteristics**

| No. | Age /Sex | Histology | PS | Stage | Site of metastases | Previous treatments | | | No. of vaccination |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Surgery | Chemotherapy[1] | Radiotherapy | |
| EBL-001 | 65 F | Adenocarcinoma | 0 | T3N0M1 IV | costal bone | – | CPT11/IFM TXT | – | 26 |
| EBL-002 | 61 M | Adenocarcinoma | 0 | T4N3M1 IV | scalp bone abdominal LNs[2] | – | – | – | 23 |
| EBL-003 | 59 M | Adenocarcinoma | 2 | Recurrent case | kidney/adrenal spinal bone inguinal LNs | lt. lower lobectomy (T2N2M0) | CDDP GEM | + mediastinal LNs | 4 |
| EBL-004 | 66 F | Adenocarcinoma | 0 | Recurrent case | lung (meta.[3]) costal bone | lt. upper lobectomy (T1N0M0) | – | + costal bone | 9 |
| EBL-005 | 69 M | Adenocarcinoma | 0 | Recurrent case | lung (recurrence[4]) lung (meta.) | lt. upper lobectomy (TxN1M0) | – | – | 29 |
| EBL-006 | 71 M | Squamous cell carcinoma | 1 | Recurrent case | lung (recurrence) lung (meta.) | rt. upper lobectomy (T2N0M0) | – | – | 16 |
| EBL-007 | 61 F | Adenocarcinoma | 0 | T4N1M1 IV | clavicular bone | – | GEM VNB | + clavicular bone | 5 |
| EBL-008 | 60 F | Adenocarcinoma | 0 | Recurrent case | lung (recurrence) | rt. upper lobectomy (T2N0M0) | – | – | 19 |
| EBL-009 | 63 F | Adenocarcinoma | 0 | Recurrent case | lung (meta.) | lt. lower lobectomy (T1N2M0) | TXL CBDCA | – | 15 |
| EBL-010 | 74 M | Small cell carcinoma | 1 | T2N3M0 IIIb | — | – | CDDP VP-16 | – | 3 |

*1*) CPT11, irinotecan hydrochloride; IFM, ifosfamide; TXT, docetaxel hydrate; CDDP, cisplatin; GEM, gemcitabine hydrochloride; VNB, vinorelbine ditartrate; TXL, paclitaxel; CBDCA, carboplatin; VP-16, etoposide.
*2*) LNs, lymph nodes. *3*) Metastatic tumors. *4*) Local recurrence.

**Table 2. Peptide and screening of the peptide-specific CTL precursor cells**

| Peptide | | Patient's No. | | | | | | | | | | Positive case | Vaccinated case |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Name | Sequence | 001 | 002 | 003 | 004 | 005 | 006 | 007 | 008 | 009 | 010 | | |
| SART1 690 | EYRGFTQDF | A[1] | | | | +[2]/D | +/D | +/A | | +/AC[3] | | 5/10 | 4/10 |
| SART2 93 | DYSARWNEI | A | | +/A | | +/A | | | +/D | | +/CC | 5/10 | 4/10 |
| SART2 161 | AYDFLYNYL | C | | | +/D | +/A | | | +/A | | +/A | 5/10 | 4/10 |
| SART2 899 | SYTRLFLIL | A | | +/B | | | | +/C | | | +/AC | 4/10 | 3/10 |
| SART3 109 | VYDYNCHVDL | +/AA | | | | | | +/Ar | | | +/B | 3/10 | 3/10 |
| SART3 315 | AYIDFEMKI | +/AA | +/CC | | | +/C | +/ArAC[3] | | | +/B | | 5/10 | 5/10 |
| CypB 84 | KFHRVIKDF | –[4]/A | | | | | | | | | | 2/10 | 0/10 |
| CypB 91 | DFMIQGGDF | A | | +/Ar | | | +/D | | | | | 3/10 | 2/10 |
| lck 208 | HYTNASDGL | +/A | +/CC | +/AA | | | | C | +/A | | | 5/10 | 4/10 |
| lck 486 | TFDYLRSVL | +/A | +/C | C | | | | | | | | 3/10 | 2/10 |
| lck 488 | DYLRSVLEDF | | | | +/B | | | +/A | +/A | +/B | | 4/10 | 4/10 |
| ART1 170 | EYCLKFTKL | | | | –/C | | –/C | | –/A | | B | 4/10 | 0/10 |
| ART4 13 | AFLRHAAL | | +/C | | | | | | | | | 1/10 | 1/10 |
| ART4 75 | DYPSLSATDI | | | | +/C | | | | | | | 1/10 | 1/10 |

*1*) The peptide-specific CTL precursor cells were evaluated by quadruplicate assay under the following criteria: Ar, peptide-specific IFN-γ production≥500 pg/ml, and *P* value<0.10; A, 50≤IFN-γ production<500 pg/ml, and *P* value<0.05; B, 25≤IFN-γ production<50 pg/ml, and *P* value<0.05; C, IFN-γ production≥50 pg/ml, and 0.05≤*P* value<0.10; D, IFN-γ production>100 pg/ml, and 0.10≤*P* value<0.20.
*2*) Plus(+)-mark: the peptide was vaccinated.
*3*) "AC" means that one well is criteria A, the other well is criteria C, and the remaining two wells are negative. "ArAC" means that three of four wells are positive containing criteria Ar, criteria A, and criteria C.
*4*) Minus(–)-mark: the peptide was not vaccinated due to immediate-type hypersensitivity by skin test.

patients; and $ART4_{13}$ and $ART4_{75}$ for 1 patient.

**Adverse events.** All patients were evaluated for adverse events, and the overall results are shown in Table 3. The most common adverse events were inflammatory reactions at the injection site, as follows: local erythema and induration (grade 1) in 5 patients; rubor and swelling (grade 2) in 2 patients; and vesicle (grade 2) in 1 patient. Although the time and duration of their appearances varied among patients, there was a tendency for these local reactions to be observed from the 3rd to 6th vaccination periods rather than in the initial three periods. In 1 patient (EBL-001), a vesicle (grade 2) was observed once at the injection site of $SART2_{161}$ at the time of the 14th vaccination. Gastrointestinal events were observed in 4 patients, including appetite loss with nausea in 1 patient (grade 1) and diarrhea

**Table 3. Adverse events**

| Category | | Grade 1 | Grade 2 | Grade 3 | Total cases |
|---|---|---|---|---|---|
| Constitutional symptoms | Fatigue | 2 | | | 2/10 |
| Dermatology/Skin | Injection site reaction | 5 | 3[1)] | | 8/10 |
| Gastrointestinal | Anorexia | 1 | | | 1/10 |
| | Colitis | | 1 | | 1/10 |
| | Diarrhea | 2 | | | 2/10 |
| | Nausea | 1 | | | 1/10 |
| Pulmonary | Dyspnea | | 1[2)] | | 1/10 |

*1)* Rubor and swelling in 2 patients, and vesicle in 1 patient.
*2)* Dyspnea with wheezing.

**Table 4. Peptide-specific immune responses and clinical responses**

| Pt. No. | Peptide | Peptide-specific CTLp[1)] Pre | Peptide-specific CTLp[1)] Post | Antibody to peptide Pre | Antibody to peptide Post | DTH Pre | DTH Post | Clinical response at 2 month | Clinical response at 6 month | TTP (days) | OS (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EBL-001 | SART3 109 | AA | ArAC | + (1.61)[2)] | + (1.68) | – | +/8th | SD | SD | 204 | 784+ |
| | SART3 315 | AA | — | + (0.51) | + (0.53) | – | – | | | | |
| | Ick 208 | A | — | – | – | – | – | | | | |
| | Ick 486 | A | — | – | + (0.06) | – | – | | | | |
| EBL-002 | SART3 315 | CC | A | – | – | – | – | MR | SD | 323 | 668 |
| | Ick 208 | CC | — | – | + (0.44) | – | – | | | | |
| | Ick 486 | C | — | – | – | – | – | | | | |
| | ART4 13 | C | — | – | – | – | – | | | | |
| EBL-003 | SART2 93 | A | NT | – | – | – | – | PD | dead | 42 | 115 |
| | SART2 899 | B | NT | – | – | – | – | | | | |
| | CypB 91 | Ar | NT | – | – | – | – | | | | |
| | Ick 208 | AA | NT | – | – | – | – | | | | |
| EBL-004 | SART2 161 | D | — | – | – | – | – | SD | SD | 196 | 258 |
| | Ick 488 | B | B | – | – | – | – | | | | |
| | ART4 75 | C | — | – | – | – | – | | | | |
| EBL-005 | SART1 690 | D | — | – | – | – | – | SD | SD | 259 | 602+ |
| | SART2 93 | A | ArAAA | – | – | – | – | | | | |
| | SART2 161 | A | AC | – | + (0.05) | – | – | | | | |
| | SART3 315 | C | A | – | + (0.04) | – | – | | | | |
| EBL-006 | SART1 690 | D | ArArA | – | – | – | – | SD | PD | 108 | 457 |
| | SART3 315 | ArAC | ArArAA | – | – | – | +/2nd | | | | |
| | CypB 91 | D | — | – | – | – | – | | | | |
| EBL-007 | SART1 690 | A | NT | – | – | – | – | SD | PD | 69 | 193 |
| | SART2 899 | C | NT | – | – | – | – | | | | |
| | SART3 109 | Ar | NT | – | – | – | – | | | | |
| | Ick 488 | A | NT | – | – | – | – | | | | |
| EBL-008 | SART2 93 | D | — | – | – | – | – | SD | PD | 147 | 538+ |
| | SART2 161 | A | — | – | – | – | – | | | | |
| | Ick 208 | A | — | – | + (0.09) | – | – | | | | |
| | Ick 488 | A | — | – | – | – | – | | | | |
| EBL-009 | SART1 690 | AC | — | – | – | – | – | PD | PD | 30 | 441+ |
| | SART3 315 | B | CD | – | – | – | +/3rd | | | | |
| | Ick 488 | B | — | – | – | – | – | | | | |
| EBL-010 | SART2 93 | CC | NT | – | – | – | – | PD | PD | 28 | 213 |
| | SART2 161 | A | NT | – | – | – | – | | | | |
| | SART2 899 | AC | NT | – | – | – | – | | | | |
| | SART3 109 | B | NT | + (0.03) | + (0.03) | – | – | | | | |

OS, overall survival; NT, not tested; SD, stable disease; MR, minor response; PD, progressive disease.
*1)* The peptide-specific CTL precursor cells were evaluated by quadruplicate assay. "AA" means that two wells are positive (criteria A) and the remaining two wells are negative.
*2)* Values indicate the optical density of sera (×100 dilution).

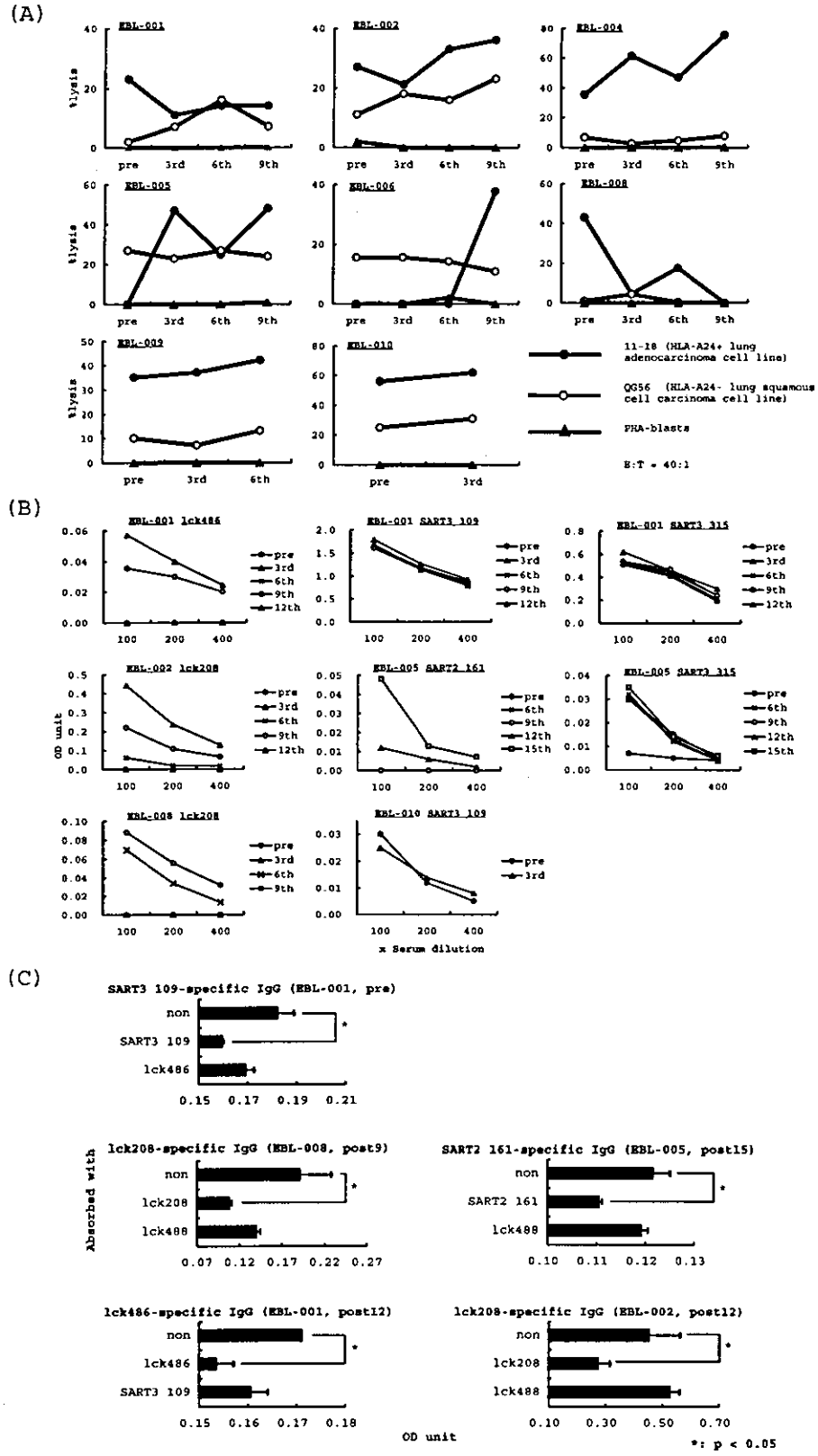


Fig. 2. Immune responses. (A) Cytotoxicity: pre- and post (3rd, 6th, and 9th)-vaccination PBMCs were cultured with IL-2 (100 U/ml) alone for 21 days. Cytotoxicity against HLA-class I-matched (11-18) and mis-matched (QG56) lung cancer cell lines and HLA-A24$^+$ PHA blastoid cells as a negative control was measured by a 6-h $^{51}$Cr release assay at an E/T ratio of 40:1 in triplicate assays. The mean of specific lysis is shown in the figure. (B) Induction and specificity of peptide-specific IgG antibodies: pre- and post (3rd, 6th, 9th, and 12th)-vaccination sera were provided for the assay, and positive results of anti-peptide IgG antibodies in 5 patients are shown in this figure. There were no detectable levels of peptide-reactive IgG in the other 5 patients. (C) Sera were absorbed with the corresponding peptide or an irrelevant peptide, followed by measurement of the peptide-specific IgG.

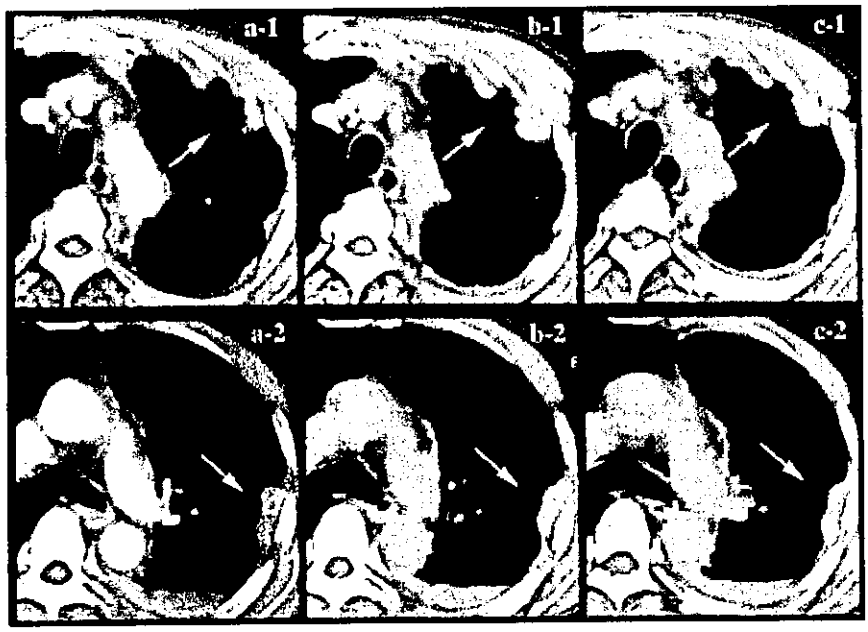


Fig. 3. CT images of the EBL-002 patient. (a) After the 1st vaccination. (b) After the 4th vaccination, the pleural dissemination was increased. (c) After the 8th vaccination, a regression of the pleural dissemination was observed.

in 2 patients (grade 1). Localized erosive colitis (grade 2) in the sigmoid colon of another patient (EBL-002) was observed with diarrhea after the 4th vaccination and hematochezia after the 8th vaccination by colonoscopy. These symptoms were diminished after dose reduction (the 12th vaccination) without medicine (steroid, immunosuppressant, or anti-inflammatory agent), and edema was only observed in the endoscopic findings for the 14th vaccination. Wheezing was observed in 1 patient (EBL-007) with a history of bronchial asthma. No medication was necessary for any of these adverse events.

**Immune responses and serum IgG levels.** DTH was observed in 3 patients (to $SART3_{109}$ after the 8th vaccination in EBL-001, to $SART3_{315}$ after the 2nd in EBL-006, and after the 3rd in EBL-009).

For evaluation of cellular immune responses to the vaccinated peptides, the post (6th)-vaccination PBMCs were provided for the screening of the peptide-specific CTL precursor assay. Two representative cases are shown in Fig. 1B. All four wells of the post-vaccination PBMCs of 1 patient (EBL-005) responded to the $SART2_{93}$ peptide with the criteria of Ar, A, A, and A; two wells responded to $SART2_{161}$ with the criteria of A and C; and one each responded to $SART1_{690}$ with C or to $SART3_{315}$ with A, respectively. Similarly, 4, 3, and 2 wells of the post-vaccination PBMCs of the other patient (EBL-006) responded to the $SART3_{315}$, $SART1_{690}$, and $ART1_{170}$ peptides, respectively. An overall summary is given in Table 4. Of the 7 patients tested, the post (6th)-vaccination PBMCs of 3 patients (EBL-001, -005, and -006) showed an increased response to at least one of the vaccinated peptides based on both the number of positive wells and criteria. In contrast, those of the other 4 patients had decreased responses.

The pre- and post (3rd, 6th, and 9th)-vaccination PBMCs were expanded *in vitro* in culture with IL-2 alone for 21 days, followed by measurement of their cytotoxicity with a 6-h $^{51}Cr$ release assay against HLA-A24$^+$ 11-18 lung adenocarcinoma cells, HLA-A24$^-$ QG56 lung squamous cell carcinoma cells, and HLA-A24$^+$ PHA blastoid cells as normal cells (Fig. 2A). No cytotoxicity was detectable in any sample from the 2 patients (EBL-003 and -007) (data not shown). Post-vaccination PBMCs from 1 patient (EBL-008) showed decreased HLA-A24-restricted cytotoxicity. No significant changes were noted in the 4 patients (EBL-001, -002, -009, and -010). Post-vaccination PBMCs of the remaining 3 patients (EBL-004 from the 3rd to 9th, EBL-005 at the 3rd and the 9th, and EBL-006 at the 9th vaccination) showed increased levels of HLA-A24-restricted cytotoxicity.

Humoral immune responses to the vaccinated peptides were measured in both pre- and post (3rd, 6th, and 9th)-vaccination sera (Fig. 2B). IgG antibodies reactive to $SART3_{109}$ and $SART3_{315}$ peptides were detected in the pre-vaccination sera of 1 patient (EBL-001), while IgG reactive to $SART3_{109}$ peptide were also detectable in the pre-vaccination sera of the other patient (EBL-010). These levels were mostly consistent throughout the vaccination period. IgG reactive to $lck_{486}$ or $lck_{208}$ peptide were induced in the post-vaccination sera of EBL-001 or EBL-002 and EBL-008 patients, respectively. Similarly, IgG reactive to $SART2_{161}$ and $SART3_{315}$ peptides was induced in sera of the EBL-005 patient. In contrast, there were no detectable levels of peptide-reactive IgG in either pre- or post-vaccination sera of the remaining 5 patients. A summary is given in Table 4. Peptide-specificity was confirmed by the absorption test (Fig. 2C). Reactivity to each of these peptides was significantly decreased by absorption with the beads coupled with the corresponding peptide, but not by those coupled with an irrelevant peptide. Collectively, peptide-specific humoral immune-responses were induced in the post-vaccination sera of the 4 patients (EBL-001, -002, -005, and -008).

**Clinical responses and prognostic factor analysis.** A summary of clinical responses is given in Table 4. Objective tumor regression was observed in only 1 patient (EBL-002). This patient had a minor response (MR), with lesions of the pleural dissemination being reduced at the 8th vaccination (Fig. 3). Of the 10 patients eligible for evaluation, 8 were diagnosed with stable disease (SD), and the remaining 2 (EBL-003 and -010) were diagnosed with progressive disease (PD) at 2 months after the first vaccination. Five of 10 patients were diagnosed with SD and the remaining 5 with PD at the 6-month evaluation. The median time to progression (TTP), the median survival time (MST), and the 1-year survival rate of 10 patients were 108.0±61.7 (±standard error) days, 457.0±186.7 days, and

60.0%, respectively. That of 9 patients with non-small cell lung cancer (NSCLC) was 147.0±65.6 days, 668.0±164.2 days, and 66.7%, respectively.

## Discussion

We previously showed that CTL precursors to EBV-derived peptide or tumor antigen-derived peptides were detectable in pre-vaccination PBMCs of healthy donors and pancreatic cancer patients, respectively.[19,20] We initially confirmed in this study that these CTL precursors were also detectable in pre-vaccination PBMCs of advanced lung cancer patients who participated in the CypB vaccination regimen from 1999 to 2000.[17] These retrospective studies demonstrated that CTL precursors to the vaccinated peptide ($CypB_{91}$) were detectable in pre-vaccination PBMCs in only 3 of 6 patients. TTP and overall survival of the CTL precursor-positive cases (shown in Fig. 1A) were longer than those of the negative three cases (data not shown). This heterogeneity of T-cell reactivity in pre-vaccination PBMCs was confirmed in 10 patients enrolled in the new regimen. All these results indicate that profiles of CTL precursors in pre-vaccination PBMCs of advanced lung cancer patients are different from each other.

Increased cellular responses to some of the vaccinated peptides were observed in the post-vaccination PBMCs in only 3 of the 7 patients tested. Similarly, increased CTL responses to HLA-A24$^+$ lung cancer cells were observed in these PBMCs in 4 of 10 patients tested. Further, increased humoral responses to some of the vaccinated peptides were observed in the post-vaccination sera in 4 of 10 patients tested. Although response rates to this new regimen were relatively low, such responses were scarcely observed in the post-vaccination PBMCs of patients receiving $CypB_{91}$ peptide under the previous regimen without pre-vaccination measurement of CTL precursors.[17] Therefore, the present regimen seems to be superior to the previous regimen with regard to induction of immune responses to both peptides and tumor cells. Further studies, however, should be carried out to determine whether a CTL precursor-oriented vaccine is better than the non-oriented vaccine, using a new regimen such as a prospective clinical study, which is now in progress at our hospital for gynecological cancers.

There was no increase in the cellular responses to any peptides in 4 of 7 patients tested (EBL-002, -004, -008, and -009), regardless of the pre-vaccination measurement of CTL precursors, whereas some of the peptides among the vaccinated peptides induced augmented responses in the remaining 3 patients (EBL-001, -005, and -006). This failure might be in part due to the inappropriate selection of peptides based on criteria B, C, or D as increased cellular responses to the peptides were observed when the peptide was selected primarily with regard to criteria A or Ar. This failure might also in part be due to the alteration of CTL precursors in the circulation. The CTLs activated by the vaccination might infiltrate into various tissues, including tumors, and induce immune responses to tumor cells, which in turn could be responsible for the appearance of CTL precursors reactive to other peptides. Limited sensitivity as well as reproducibility of this CTL assay could be involved in this failure.

The requirement of CD4 T cells to help with initiating and sustaining a CD8 response is well established in animal models,[25] although the exact role of the CD4 T cells in regression of human lung cancers remains unknown at the present time. We previously reported that IgG reactive to the SART3 peptide or CypB peptide became detectable in the post-vaccination sera of colon cancer patients[18] or lung cancer patients,[17] respectively. This study confirmed the previous results. The role of these antibodies in anti-tumor immunity is unclear, although the therapeutic effectiveness of humanized monoclonal antibodies, such as anti-HER2/neu (Herceptin), on tumor regression is now widely accepted.[28–30] Therefore, the various roles of these peptide-specific IgG antibodies in tumor-regression need to be determined in future basic and clinical studies. Mechanisms of IgG production, including the involvement of CD4 T helper cells and MHC restriction also need to be clarified.

One of our objectives was to find an appropriate immunological marker for prediction of clinical course in response to vaccination. Although only 10 patients were enrolled in this small-scale phase I study with limited observation periods, measurement of peptide-specific IgG might be a marker for prediction of good clinical responses, as all 4 patients with increased levels of peptide-specific IgG had a long progression-free survival (median TTP was 204.0±56.0 days, and that of the remaining 6 patients was 42.0±23.9 days; logrank test, $P$=0.0119) and a long overall survival (MST was 668.0±472.3 days including three surviving cases, and that of the remaining 6 patients was 213.0±39.8 days; logrank test, $P$=0.0110). In contrast, DTH responses did not show any apparent correlation with clinical course. Neither cellular immune response to the peptide nor that to HLA-A24$^+$ tumor cells showed a clear correlation with clinical course. Cytotoxicity against either HLA-A24$^+$ or HLA-A24$^-$ lung cancer cells was not detectable in any samples of the 2 patients (EBL-003 and -007) with TTPs of 42 and 69 days, respectively. Their TTPs were the shortest among the 9 NSCLC patients. These results suggest that lack of tumor cell lysis is a marker for prediction of a poor clinical course. Further studies with a larger numbers of cases, however, are required to test these ideas.

None of the 10 patients showed a major regression, although EBL-002 had a minor regression of the pleural dissemination. However, median TTP and MST of these 10 patients seem to be not inferior to those of advanced lung cancer patients treated with various types of chemotherapies. In particular, the 9 patients with advanced NSCLC showed the MST of 668.0±164.2 days and the 1-year survival rate of 66.7%. The prognosis of advanced lung cancer patients is very poor. A large sample of phase III trials with chemotherapy was associated with MST of 8 to 10 months and a 1-year survival rate of 30 to 35%.[31] We know that these benefits should not be compared with those of any treatment, since this study was a phase I trial. However, this new regimen might have potential benefits for advanced NSCLC patients.

It should therefore be determined in a phase II clinical study whether a regimen of CTL precursor-oriented peptide vaccine can provide clinical benefits for advanced lung cancer patients.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sport, Science and Technology and from the Ministry of Health, Labour and Welfare, Japan.

1. Van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; **254**: 1643–7.
2. Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannelli JR, Appella E, Rosenberg SA. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J Exp Med* 1994; **180**: 347–52.
3. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, Yannelli JR, Adema GJ, Miki T, Rosenberg SA. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. *Proc Natl Acad Sci USA* 1994; **91**: 6458–62.
4. Brichard V, Van Pel A, Wolfel T, Wolfel C, De Plaen E, Lethe B, Coulie P, Boon T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med* 1993; **178**: 489–95.

5. Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, Rosenberg SA. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med* 1996; **183**: 1185–92.
6. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci USA* 1995; **92**: 432–6.
7. Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* 1995; **181**: 2109–17.
8. Correale P, Walmsley K, Nieroda C, Zaremba S, Zhu M, Schlom J, Tsang KY. *In vitro* generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. *J Natl Cancer Inst* 1997; **89**: 293–300.
9. Correale P, Walmsley K, Zaremba S, Zhu M, Schlom J, Tsang KY. Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligoepitope peptide. *J Immunol* 1998; **161**: 3186–94.
10. Jager E, Gnjatic S, Nagata Y, Stockert E, Jager D, Karbach J, Neumann A, Rieckenberg J, Chen YT, Ritter G, Hoffman E, Arand M, Old LJ, Knuth A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. *Proc Natl Acad Sci USA* 2000; **97**: 12198–203.
11. Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Lienard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jager E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, van der Bruggen P, Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer* 1999; **80**: 219–30.
12. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 1998; **4**: 321–7.
13. Valmori D, Dutoit V, Rubio-Godoy V, Chambaz C, Lienard D, Guillaume P, Romero P, Cerottini JC, Rimoldi D. Frequent cytolytic T-cell responses to peptide MAGE-A10(254-262) in melanoma. *Cancer Res* 2001; **61**: 509–12.
14. Gajewski TF, Fallarino F, Ashikari A, Sherman M. Immunization of HLA-A2+ melanoma patients with MAGE-3 or MelanA peptide-pulsed autologous peripheral blood mononuclear cells plus recombinant human interleukin 12. *Clin Cancer Res* 2001; **7**: 895s–901s.
15. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Gladhaug I, Soreide O, Eriksen JA, Moller M, Baksaas I, Lothe RA, Saeterdal I, Gaudernack G. Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: clinical and immunological responses in patients with pancreatic adenocarcinoma. *Int J Cancer* 2001; **92**: 441–50.
16. Lau R, Wang F, Jeffery G, Marty V, Kuniyoshi J, Bade E, Ryback ME, Weber J. Phase I trial of intravenous peptide-pulsed dendritic cells in patients with metastatic melanoma. *J Immunother* 2001; **24**: 66–78.
17. Gohara R, Imai N, Rikimaru T, Yamada A, Hida N, Ichiki M, Kawamoto M, Matsunaga K, Ashihara J, Yano S, Tamura M, Ohkouchi S, Yamana H, Oizumi K, Itoh K. Phase 1 clinical study of cyclophilin B peptide vaccine for lung cancer patients. *J Immunother* 2002; **25**: 439–44.
18. Miyagi Y, Imai N, Sasatomi T, Yamada A, Mine T, Katagiri K, Nakagawa M, Muto A, Okouchi S, Isomoto H, Shirouzu K, Yamana H, Itoh K. Induction of cellular immune responses to tumor cells and peptides in colorectal cancer patients by vaccination with SART3 peptides. *Clin Cancer Res* 2001; **7**: 3950–62.
19. Hida N, Maeda Y, Katagiri K, Takasu H, Harada M, Itoh K. A simple culture protocol to detect peptide-specific cytotoxic T lymphocyte precursors in the circulation. *Cancer Immunol Immunother* 2002; **51**: 219–28.
20. Suzuki N, Maeda Y, Tanaka S, Hida N, Mine T, Yamamoto K, Oka M, Itoh K. Detection of peptide-specific cytotoxic T lymphocyte precursors used for specific immunotherapy of pancreatic cancer. *Int J Cancer* 2002; **98**: 45–50.
21. Shichijo S, Nakao M, Imai Y, Takasu H, Kawamoto M, Niiya F, Yang D, Toh Y, Yamana H, Itoh K. A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. *J Exp Med* 1998; **187**: 277–88.
22. Nakao M, Shichijo S, Imaizumi T, Inoue Y, Matsunaga K, Yamada A, Kikuchi M, Tsuda N, Ohta K, Takamori S, Yamana H, Fujita H, Itoh K. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. *J Immunol* 2000; **164**: 2565–74.
23. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, Mori K, Hayashi A, Yamana H, Shirouzu K, Itoh K. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. *Cancer Res* 1999; **59**: 4056–63.
24. Gomi S, Nakao M, Niiya F, Imamura Y, Kawano K, Nishizaka S, Hayashi A, Sobao Y, Oizumi K, Itoh K. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. *J Immunol* 1999; **163**: 4994–5004.
25. Harashima N, Tanaka K, Sasatomi T, Shimizu K, Miyagi Y, Yamada A, Tamura M, Yamana H, Itoh K, Shichijio S. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. *Eur J Immunol* 2000; **31**: 323–32.
26. Nishizaka S, Gomi S, Harada K, Oizumi K, Itoh K, Shichijo S. A new tumor-rejection antigen recognized by cytotoxic T lymphocytes infiltrating into a lung adenocarcinoma. *Cancer Res* 2000; **60**: 4830–7.
27. Kawano K, Gomi S, Tanaka K, Tsuda N, Kamura T, Itoh K, Yamada A. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A24-restricted tumor infiltrating lymphocytes of lung cancer. *Cancer Res* 2000; **60**: 3550–8.
28. Leyland-Jones B. Trastuzumab: hopes and realities. *Lancet Oncol* 2002; **3**: 137–44.
29. Rosen LS. Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. *Cancer Control* 2002; **9**: 36–44.
30. Czuczman MS. Immunochemotherapy in indolent non-Hodgkin's lymphoma. *Semin Oncol* 2002; **29**: 11–7.
31. Socinski MA, Morris DE, Masters GA, Lilenbaum R. Chemotherapeutic management of stage IV non-small cell lung cancer. *Chest* 2003; **123**: 226s–43s.

# Identification of a CTL-directed epitope encoded by an intron of the putative tumor suppressor gene Testin of the common fragile site 7G region: a peptide vaccine candidate for HLA-B52$^+$ and HLA-62$^+$ cancer patients

**Shinya Ohkouchi[1,3], Naoki Kawamoto[1], Makoto Koga[1], Fumi Sakanashi[1], Shigeki Shichijo[2], Yasuo Saijo[3], Toshihiro Nukiwa[3], Kyogo Itoh[1,2] and Akira Yamada[1,2]**

[1] Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, Kurume, Japan
[2] Department of Immunology, Kurume University School of Medicine, Kurume, Japan
[3] Respiratory Oncology and Molecular Medicine, Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan

Although cytotoxic T lymphocyte (CTL)-directed epitopes binding to human histocompatibility leukocyte antigen (HLA)-A molecules have been well characterized, those binding to HLA-B molecules have not, largely due to their large diversity. In this study we report a unique cancer antigen gene, tentatively named Testin-related gene (TRG), which encodes CTL-directed epitopes on the HLA-B52 molecules most frequently expressed in Asians. TRG is located in an intron of the putative tumor suppressor gene Testin in the common fragile site 7G region at 7q31.2. TRG mRNA was expressed in the majority of cancer cells and cancer tissue tested, whereas it was scarcely expressed in the majority of normal tissues, and only low-level expression of TRG was detected in the heart, liver, and pancreas. One TRG peptide had the ability to induce HLA-B52-restricted CTL cytotoxic to TRG$^+$ tumor cells in peripheral blood mononuclear cells (PBMC) of epithelial cancer patients. This peptide also induced HLA-B62-restricted and tumor-reactive CTL in PBMC of cancer patients. Therefore, this TRG-derived peptide might be appropriate for use in peptide-based immunotherapy for relatively large numbers of cancer patients throughout the world, given that 34% of Japanese, 27% of Chinese, and 13% of Caucasians express either HLA-B52 or HLA-B62 molecules

| | |
|---|---|
| Received | 5/5/03 |
| Revised | 7/8/03 |
| Accepted | 25/8/03 |

**Key words:** New antigen / CTL epitope / HLA-B52 / Cancer vaccine / Testin

## 1 Introduction

The current standard cancer therapies are not very effective for advanced or metastatic cancers [1]. Among the epithelial cancers, lung cancer is the highest cause of cancer death in many countries [2–5]. Because the incidence of lung cancer is increasing, there is an urgent need for more effective approaches with fewer adverse events. To this end, specific immunotherapy could be a powerful strategy [6]. Although relatively large numbers of CTL-directed epitopes binding to HLA-A molecules were identified from the cDNA of melanomas and other epithelial cancers [7–17], only a few CTL-directed epitopes binding to HLA-B molecules have been identified [18–26]. This is hampering the clinical trials of specific cancer therapy with HLA-B allele-binding peptides.

One of the major difficulties regarding HLA-B alleles is their large diversity, whereas several HLA-A alleles, such as HLA-A2 and HLA-A24, are widely common among the different ethnic groups. HLA-B52, however, is one of the most frequently expressed B alleles in different ethnic groups (34% of Japanese, 27% of Chinese, and 13% of Caucasians) [27]. In the present study, we therefore attempted to identify CTL-directed epitopes of lung adenocarcinoma cells that were recognized by HLA-B52-restricted CTL, and to report a new cancer antigen, the Testin-related gene (TRG) protein, that is preferentially expressed in epithelial cancer cells.

[DOI 10.1002/eji.200324149]

**Abbreviations: TRG:** Testin-related gene **HD:** Healthy donor

## 2 Results

### 2.1 Identification and characterization of the gene

The HLA-B5201-restricted and tumor-specific CTL (GK-B-CTL) line with a $CD3^+CD4^-CD8^+$ phenotype was established by long-term incubation with IL-2 alone of tumor-infiltrating lymphocytes from a patient with lung adenocarcinoma (HLA-A0206/2402, B39/52, Cw7). This GK-B-CTL line produced significant levels of IFN-γ in response to 11–18 (HLA-A0201/2402, B5201/54, C0102/1202) and Sq-1 (HLA-A1101/2402, B5201/1501, C0102), but not in response to PC9 (HLA-A0206/2402, B0702/5502, C0303/0702) or any other HLA-$B5201^-$ cells tested (Table 1). The CTL activity was inhibited by anti-HLA class I (ABC) and anti-HLA-BC mAb (Fig. 1), but not by any other mAb tested (data not shown), suggesting an HLA-B restriction of the activity. Subsequently, cells of this CTL line were used as indicator cells in the following experiments.

A total of $10^5$ cDNA clones from the cDNA library of the 11–18 lung adenocarcinoma cell line were tested for their ability to stimulate IFN-γ production by GK-B-CTL after cotransfection with HLA-B5201 cDNA into the COS7 cells. After repeated cycles of screening, one clone, clone 12A, was confirmed to encode a tumor antigen recognized by the GK-B-CTL when cotransfected with HLA-B5201, but not when cotransfected with the control HLA-A2601 (Fig. 2). The CTL failed to react to the cells transfected with HLA-B5201 alone or with a vacant vector, pCMVSPORT2, which served as a negative control in this experiment. Clone 12A contained a 1952-bp-long cDNA insert with seven short open reading frames encoding 32–64 amino acids (GenBank accession no. AY143171). The nucleotide sequence of clone 12A was identical to that of the intron between exons 6 and 7 of the Testin gene (accession no. AF260225). Therefore, we tentatively named clone 12A the TRG.

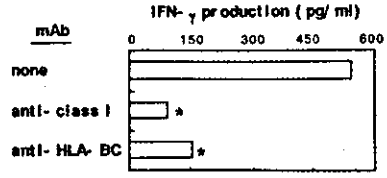


*Fig. 1.* Effect of anti-HLA mAb on INF-γ production of GK-B-CTL in response to Sq-1 lung cancer cells; $^*p<0.05$ by Student's *t*-test.

### 2.2 Expression of TRG mRNA

The expression of TRG in various tumor cell lines and ovarian cancer tissues at the mRNA level was analyzed by Northern blotting. Representative results of the Northern blot analysis are shown in Fig. 3. TRG expression with a 1.9-kb band was detectable in the majority of tumor cell lines and cancer tissues tested. In contrast, only low levels of TRG were expressed in PBMC and PHA-activated PBMC (PHA-blast). TRG expression in the normal tissues and cancer tissues was further analyzed by a quantitative real-time PCR (Table 2). Scarcely expression of TRG was again confirmed in the majority of normal tissues, and only low-level expression of TRG was detected in the heart, liver, and pancreas. In contrast, relatively high level of TRG expression was confirmed in the cancer tissues.

### 2.3 CTL epitope peptide

Each of ten different TRG-derived synthetic peptides with binding motifs for the HLA-B5201 molecule was

**Table 1.** HLA-B-restricted recognition of tumor cells by GK-B-CTL[a]

| Target cells | | | | IFN-γ production |
|---|---|---|---|---|
| name | HLA-A | HLA-B | HLA-C | (pg/ml) |
| 11–18 | 0206/2402 | 5201/5401 | 0102/1202 | 348±51[b] |
| Sq1 | 1102/2402 | 1501/5201 | 0401/1202 | 487±55[b] |
| PC9 | 0206/2402 | 0702/5502 | 0303/0702 | 95±39 |
| QG56 | 2601/– | 4601/– | 0102/– | 40±21 |
| COS-7 | – | – | – | 49±18 |

[a] HLA type of effector GK-B-CTL is A0206/A2402, B39/B52, Cw7/–.
[b] $p<0.05$ by Student's *t*-test.

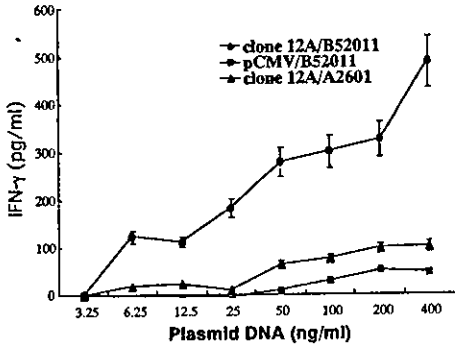


*Fig. 2.* Gene identification of a gene. COS7 cells were transfected with various doses of clone 12A or a vacant vector pCMVSPORT2.0 DNA, along with 100 ng of HLA-B5201 or control HLA-A2601 cDNA, after which their activity to stimulate IFN-γ production by the GK-B-CTL was tested. Values represent the means of triplicate assays.

loaded on HLA-B5201$^+$ SS-EBB cells at a concentration of 10 μM, and its ability to induce IFN-γ production by GK-B-CTL was tested. Detailed results are shown in Table 3. One of these peptides, TRG1–20, induced significant levels of IFN-γ production (Fig. 4A) in a dose-dependent manner (Fig. 4B). The binding affinity of TRG1–20 to the HLA-B5201 molecule was the highest among those of the tested peptides (its half-time of dissociation score was 220).

The TRG1–20 peptide was then tested for its ability to induce CTL in PBMC of ten HLA-B52$^+$ cancer patients (five with non-small-cell lung cancer, four with colon adenocarcinoma, and one with stomach adenocarcinoma) and four HLA-B52$^+$ healthy donors (HD). The PBMC were stimulated four times every 3 days by the peptide, and their ability to produce IFN-γ in response to the peptide-

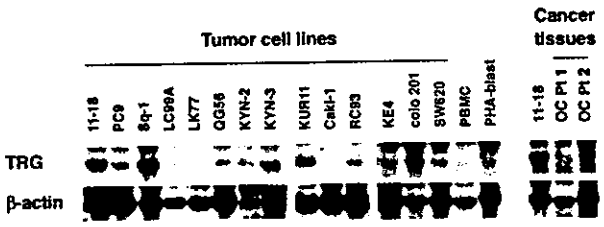


*Fig. 3.* TRG expression at the mRNA levels. TRG expression in tumor cell lines, normal tissues, and ovarian cancer (OC) tissues was investigated by Northern blot analyses. Origins of the cell lines were as follows: lung cancer (11–18, PC9, Sq-1, LC99A, LK77, QG56), hepatocellular carcinoma (KYN-2, KYN-3), renal cell carcinoma (KUR11, Caki-1, RC93), esophageal cancer (KE4), and colon cancer (colo 201, SW620).

**Table 2.** Quantitative analysis of TRG mRNA expression in normal and cancer tissues

| Tissues | TRG mRNA copies/$10^4$ β-actin copies |
|---|---|
| Normal tissues | |
| Heart | 866 |
| Brain | 482 |
| Placenta | 317 |
| Lung | 108 |
| Liver | 967 |
| Skeletal muscle | 137 |
| Kidney | 619 |
| Pancreas | 985 |
| Spleen | 347 |
| Thymus | 427 |
| Prostate | 256 |
| Testis | 673 |
| Ovary | 39 |
| Small Intestine | 39 |
| Colon | 42 |
| Leukocyte | 608 |
| Cancer tissues | |
| Breast Carcinoma | 2,495 |
| Lung Carcinoma | 3,361 |
| Lung Carcinoma | 3,917 |
| Colon Adenocarcinoma | 1,184 |
| Colon Adenocarcinoma | 6,321 |
| Prostatic Adenocarcinoma | 4,379 |

loaded SS-EBB cells or 11–18 cancer cells (HLA-B5201$^+$TRG$^+$) was examined. Significant levels of IFN-γ (>100 pg/ml) production in response to TRG1–20 peptide-loaded SS-EBB cells were detected in eight of ten cancer patients and one of four HD (Fig. 5A, left panel), and similar results were obtained in response to 11–18 tumor cells (Fig. 5A, right panel). A positive control influenza peptide successfully induced peptide-specific T cells in PBMC from six of ten patients and three of four HD, whereas a negative control HIV-peptide failed to do so at all (data not shown).