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 samples, and the values are shown as OD U/ml. To confirm the specificity of IgG to the PSCA 76–84 peptide, sample plasma was cultured with plates coated with either the PSCA 76–84 peptide or the control HIV peptide. Thereafter, the levels of PSCA 76–84 peptide-specific IgG in the resultant supernatant were determined by ELISA. To determine the sensitivity limit of the ELISA, plasma from 11 healthy donors (HIV negative) were measured for their reactivity to the HIV peptide, taken as a negative control peptide. Then, the mean + 3 SD value (0.15) was determined as the cut-off value.

Statistics

The statistical significance of the data was determined using a two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Induction of PSCA Peptide-Specific CTLs From Prostate Cancer Patients

PBMCs from 11 HLA-A24$^+$ prostate cancer patients were incubated with each of three kinds of PSCA-derived peptides or control peptides, and examined for their IFN-γ production in response to corresponding peptide-pulsed C1R-A24 cells (Table I). The assay was carried out in quadruplicate as described in Materials and Methods, and the cells showing the most pronounced IFN-γ production are shown. Successful induction of peptide-specific CTLs was judged to be positive when the *P* value was less than 0.05 and more than 50 pg/ml of IFN-γ was produced in response to the corresponding peptide-pulsed C1R-A24 cells compared to HIV peptide-pulsed C1R-A24 cells. As a result, the PSCA 76–84 peptides induced peptide-specific CTLs in six of 11 patients. The PSCA 27–36 peptide induced peptide-specific CTLs in two of 11 patients, and the PSCA 82–91 peptide failed to induce peptide-specific CTLs in any patients. These findings indicate that, among the three PSCA peptide candidates, the PSCA 76–84 is the most immunogenic peptide in HLA-A24$^+$ prostate cancer patients.

Induction of Prostate Cancer-Reactive CTLs From HLA-A24$^+$ Prostate Cancer Patients by the PSCA 76–84 Peptide

Next, it was determined whether or not PSCA 76–84 peptide-induced CTLs could show any reactivity against prostate cancer cells. The reactivity to a parental PC93 cell line and its HLA-A24-transfectant, PC93-A24, was compared in order to test HLA-A24-restricted CTL responses to prostate cancer cells. PBMCs from three HLA-A24$^+$ patients were cultured in 12-wells in the presence of the PSCA 76–84 peptide to determine whether PSCA peptide-reactive CTLs from HLA-A24$^+$ patients show reactivity against prostate cancer. As shown in (Fig. 1), PBMCs from patients #4, #8, and #11, which were in vitro stimulated with the PSCA 76–84 peptide, exhibited a higher level of cytotoxicity against PC93-A24 than against PC93, Colo320, or Colo201 (Fig. 1). We recently reported that PSCA is expressed on the surface of PC93 cells, but not on the surface of Colo320 and Colo201 colon carcinoma cells [21]. Although no peptide-specific response was observed in PBMCs of patient #4 with HRPC in the previous experiment (Table I), prostate cancer-reactive CTLs were induced from the PBMCs of this patient in this experiment. In addition, PSCA 76–84 peptide-stimulated PBMCs from these three patients significantly produced IFN-γ in response to PSCA 76–84 peptide pulsed C1R-A24 cells (data not shown). Taken together, these results indicate that PBMCs in vitro stimulated with the PSCA 76–84 peptide can show HLA-A24-restricted cytotoxicity against prostate cancer cells.

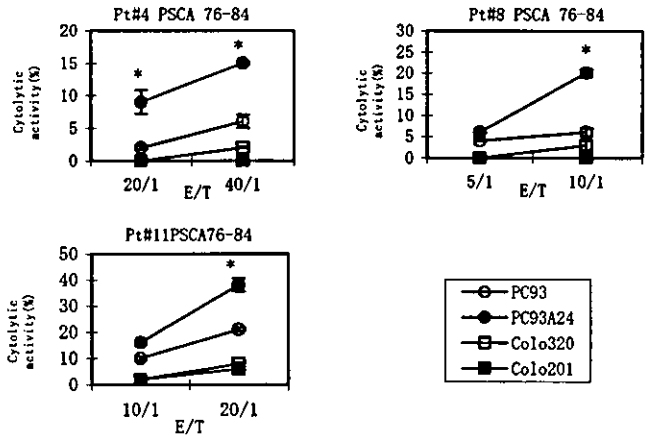

Fig. 1. Induction of HLA-A24-restricted and prostate cancer-reactive cytotoxic T lymphocytes (CTLs) from peripheral blood mononuclear cells (PBMCs) of cancer patients and healthy donors. PBMCs from three HLA-A24$^+$ prostate cancer patients (#4, #8, and #11) were in vitro stimulated with the indicated prostate stem cell antigen (PSCA) peptides in 12-wells, as described in Materials and Methods. On day 15, half of the cultured cells were harvested and confirmed their reactivity to the PSCA 76–84 peptide based on interferon (IFN)-γ production. Thereafter, a 6-hr cytotoxicity assay was performed to measure cytotoxicity against PC93, PC93-A24, HLA-A24$^+$ Colo320, and HLA-A24$^-$ Colo201. Values represent the mean of triplicate assays. *Statistically significant value at $P < 0.05$.

PSCA Peptide-Specific and CD8$^+$ T Cell-Dependent Cytotoxicity of the PSCA 76–84 Peptide-Stimulated PBMCs

It was further confirmed that cytotoxicity of the PSCA 76–84 peptide-stimulated PBMCs was dependent of CD8$^+$ T cells. As shown in Figure 2A, cytotoxicity of the PSCA 76–84 peptide-stimulated PBMCs from four patients (#4, #5, #8, and #11) was significantly inhibited by the addition of anti-class I or anti-CD8 mAb, but not by the addition of anti-class II (HLA-DR), anti-CD4, or anti-CD14 mAb. In addition, the cytotoxicity against PC93-A24 was suppressed when added with the PSCA 76–84 peptide-pulsed C1R-A24 cells, but not with the HIV peptide-pulsed C1R-A24 cells, as cold targets (Fig. 2B). These experiments with blocking antibodies and cold targets indicate that cytotoxicity of the PSCA 76–84 peptide-stimulated PBMCs was dependent on PSCA peptide-specific and CD8$^+$ T cells.

Detection of IgG Reactive to the PSCA Peptides

We have previously reported that IgGs reactive to CTL epitope peptides have been detected in patients with epithelial cancers and healthy donors [19,20]. In addition, we revealed that IgGs reactive to PSA-derived or PSMA-derived peptides that have the ability to induce CTLs were detectable in prostate cancer patients and healthy donors [22,23]. Therefore, we here attempted to determine whether IgG reactive to PSCA-derived peptides could be detected in the plasma of cancer patients and healthy donors (Table II and Fig. 3). IgG reactive to a corresponding PSCA peptide was judged to be positive when a difference of a 1:100-diluted plasma was more than 0.15, which was the mean+3 SD value as described in Materials and Methods. The result was that IgG reactive to the PSCA 76–84 peptide was detected in six patients (#1, #2, #3, #8, #12, and #13) of 11 cancer patients. Patients #12 and #13 were patients with HRPC. IgG specific to the PSCA 27–36 peptide was detected in five patients (#1, #2, #3, #5, and #8). IgG reactive to the PSCA 82–91 peptide was detected in six patients (#1, #2, #3, #5, #7, and #8). Representative results of IgG reactive to the PSCA 76–84 peptide are shown in Figure 3A. The specificity of IgG against the PSCA 76–84 peptide was confirmed by an antibody absorption test. Namely, the levels of anti-PSCA 76–84 peptide IgG in the plasma of patient #11 were significantly diminished by incubation on the PSCA 76–84 peptide-coated plate compared to incubation on the other three peptide-coated plates (Fig. 3B). We further examined the levels of IgG reactive to the PSCA 76–84 peptide in plasma of additional 12 HRPC patients, and found that IgGs to this peptide were detected in six of them (data not shown).

DISCUSSION

Prostate cancer seems to be a good target of specific immunotherapy [24]. We have been trying to identify epitope peptides that are derived from prostate-related antigens and that are able to generate prostate cancer-reactive CTLs from prostate cancer patients [10,21–23]. In treating prostate cancer patients, one of the major obstacles is the treatment of HRPC and bone metastases, because prostate cancer easily turns to be HRPC and frequently metastasizes to bone. Therefore, we tried to identify epitope peptides, which are useful in specific immunotherapy of prostate cancer patients with HRPC and/or metastases.

Because PSCA is overexpressed in both androgen-dependent and androgen-independent prostate cancers [15], and because its expression is higher in metastases than in primary tumors [16], PSCA has been considered a promising target for the treatment of prostate cancer patients. Indeed, several PSCA-derived peptides that are capable of inducing prostate cancer-reactive CTLs have been identified. The PSCA 14–22 peptide was reported to be capable of generating a PSCA-specific T cell response from an HLA-A*0201$^+$ patient with metastatic prostate cancer [25]. Both the PSCA 14–22 and PSCA 105–113 peptides were revealed to be immunogenic in HLA-A*0201$^+$ prostate cancer patients, and to induce HLA-A*0201-restricted and prostate cancer-reactive CTLs [26]. We also recently revealed that both the PSCA 7–15 and PSCA 21–30 peptides effectively induce HLA-A2-restricted and prostate cancer-reactive CTLs from prostate cancer patients [21]. These two PSCA peptides were suggested to be immunogenic in several HLA-A2 subtypes, including HLA-A*0201, -A*0206, and -A*0207. In this study, we newly identified a PSCA-derived peptide, PSCA 76–84, which is immunogenic in HLA-A24$^+$ prostate cancer patients. In particular, the PSCA 76–84 peptide induced prostate cancer-reactive CTLs from patient 4, who was diagnosed with HRPC. Totally, this information could extend the application of PSCA peptide-based vaccination for prostate cancer patients with HRPC and/or metastases.

In the clinical setting for active immunization with the PSCA 76–84 peptide, it will be valuable to determine whether or not this peptide is processed and presented on prostate cancer cells in vivo. Therefore, it should first be determined whether or not PSCA 76–84 peptide-stimulated PBMCs can show reactivity against freshly isolated PSCA$^+$ prostate cancer cells from an HLA-A24$^+$ patient. However, we have not been able to carry out this experiment because we have had difficulty obtaining large enough quantities of fresh tumor cells to form a target in ^{51}Cr-release assay. Instead, we reveal in this study that the cytotoxicity of PSCA 76–84

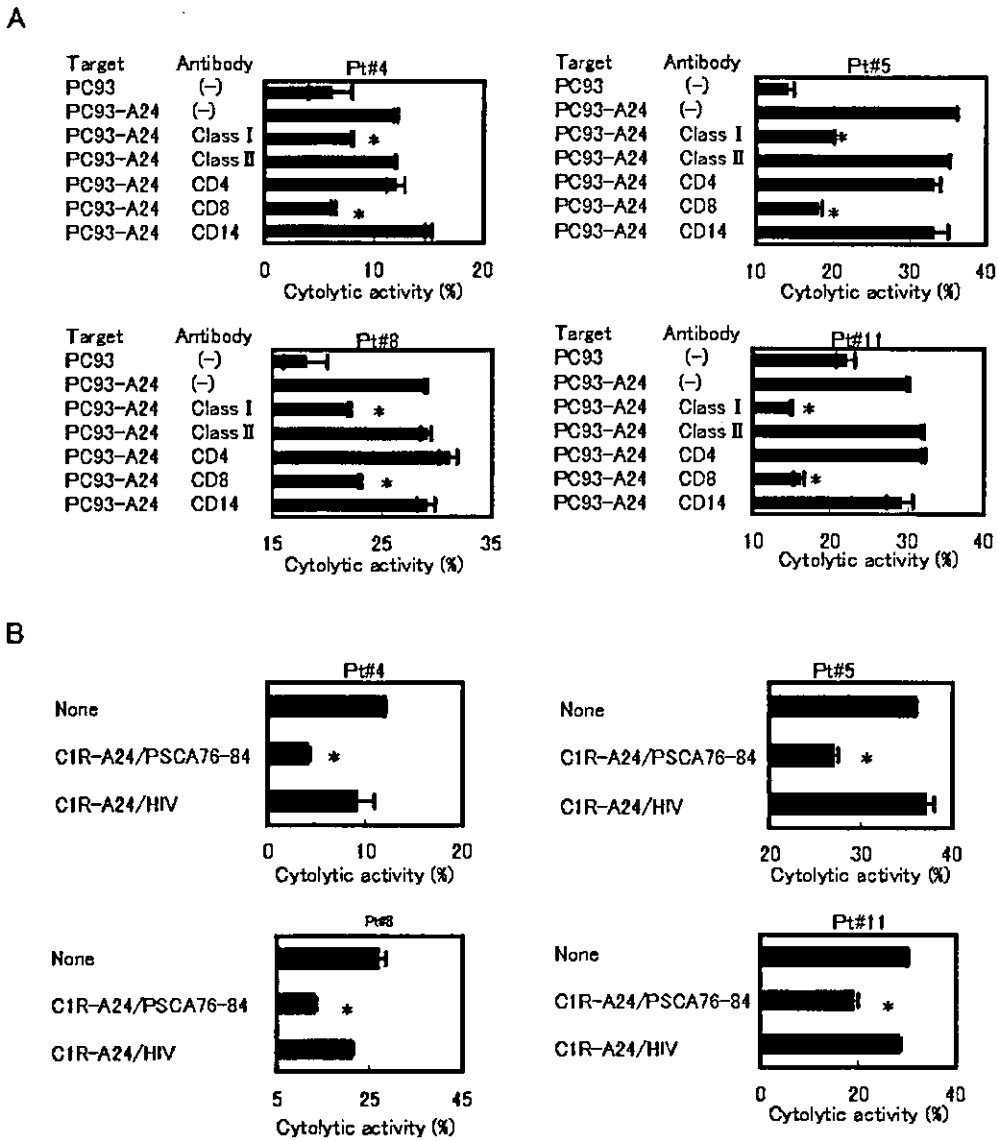

Fig. 2. $CD8^+$ T cell-dependent and PSCA 76–84 peptide-specific cytotoxicity. PBMCs from four HLA-$A24^+$ prostate cancer patient (#4, #5, #8, and #11) were in vitro stimulated with the PSCA 76–84 peptide, as described in Materials and Methods. On day 15, half of the cultured cells were harvested and confirmed their reactivity to the PSCA 76–84 peptide based on IFN-γ production. **A**: Thereafter, these PSCA peptide-stimulated PBMCs were examined for their cytotoxicity against PC93 and PC93-A24 cells with or without anti-class I, anti-class II, anti-CD4, anti-CD8, or anti-CD14 monoclonal antibody (mAb). An assay of cytotoxicity was carried out for 6 hr. Values represent the mean of triplicate assays. *Statistically significant value at $P < 0.05$. **B**: Their cytotoxicity against PC93-A24 cells was also examined in the presence of unlabeled C1R-A24 cells, which were pre-pulsed with the human immunodeficiency virus (HIV) peptide or the PSCA 76–84 peptide, as cold inhibition targets. Values represent the mean of triplicate assays. *Statistically significant value at $P < 0.05$.

peptide-induced CTLs against HLA-$A24^+$ $PSCA^+$ PC93-A24 cells was inhibited by the addition of PSCA 76–84 peptide-pulsed unlabeled C1R-A24 cells, but not by the addition of irrelevant HIV peptide-pulsed unlabeled C1R-A24 cells (Fig. 2B). We consider that this PSCA 76–84 peptide-specific decrease in cytotoxicity indirectly indicates that the PSCA 76–84 peptide could be processed and presented in prostate cancer cells.

We also assayed for the presence of IgG against PSCA peptides in plasma from HLA-$A24^+$ prostate cancer patients, since antibodies against class I-binding tumor peptides were been detected in certain cancer patients and healthy donors [19,20]. In addition, we have previously reported that IgG reactive to peptides derived from prostate-related antigens was frequently detectable in healthy donors and prostate cancer patients [21–23]. In this study, IgG reactive to any one of the three PSCA peptides was detected in 30–40% of prostate cancer patients. This means that the PSCA 76–84 peptide was recognized by both cellular and

TABLE II. IgG Reactive to the PSCA Peptides in Plasma of HLA-A24$^+$ Prostate Cancer Patients

	Cancer patients											
Peptiedes	#1	#2	#3	#4	#5	#6	#7	#8	#10	#12	#13	Total
PSCA 27–36	+	+	+	−	+	−	−	+	−	−	−	5/11
PSCA 76–84	+	+	+	−	−	−	−	+	−	+	+	6/11
PSCA 82–91	+	+	+	−	+	−	+	+	−	−	−	6/11

IgG, immunoglobulin G.
IgG reactive to a corresponding peptide was judged to be positive when a difference of optical density (OD) at a 1:100-diluted plasma was more than which was the mean + 3 SD value as described in Materials and Methods.

humoral immune systems. Although we have no clear understanding of the roles that peptide-specific IgG plays in anti-tumor immune responses, our clinical trials revealed that peptide vaccination frequently resulted in the induction of IgG reactive to administered peptides [27,28]. The induction of IgG reactive to vaccinated peptides has been positively correlated with longer survival of advanced lung cancer patients [29], and prolonged survival has been observed in gastric cancer patients showing not only cellular but also humoral immune responses to vaccinated peptides [30]. Furthermore, the induction of IgG reactive to administered peptides has been correlated with clinical responses of patients with recurrent gynecologic cancers [31]. We have been working to elucidate the roles of peptide-specific IgG in anti-tumor immune response.

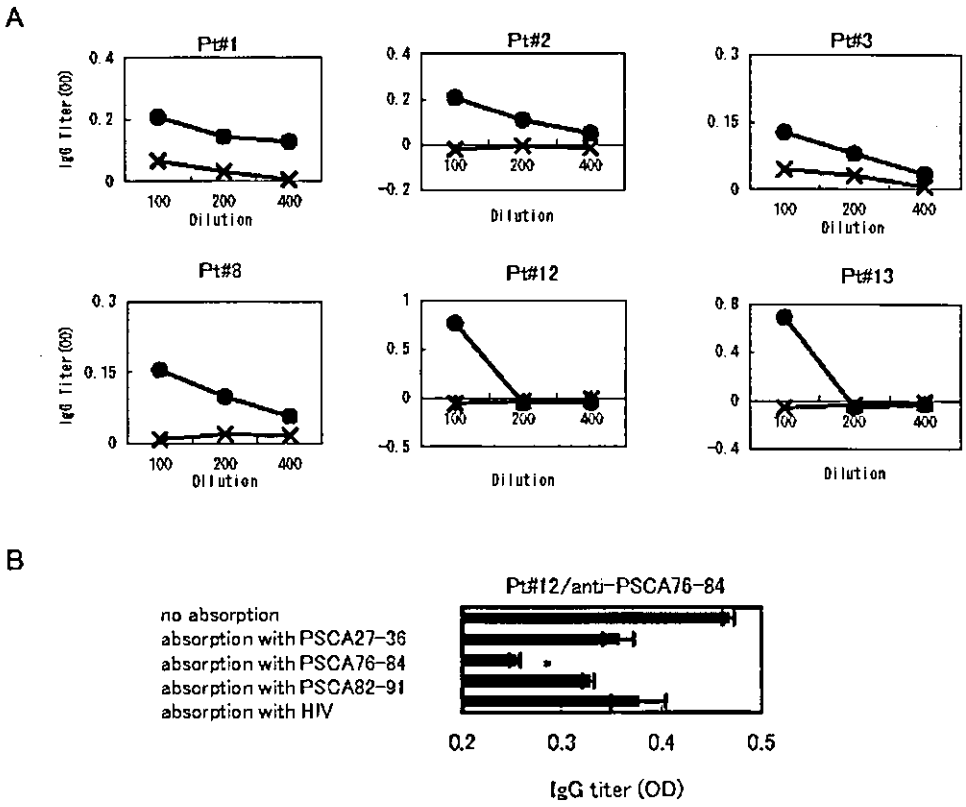

Fig. 3. Immunoglobulin G (IgG) reactive to the PSCA 76 – 84 peptide in plasma from prostate cancer patients. **A**: The positive results of IgG reactive to the PSCA 76 – 84 peptide (symbol: closed circle) are shown. These values are shown as optical density (OD) U/ml, and the HIV peptide (symbol: cross) was used as a negative control. **B**: To confirm the specificity of IgG to the PSCA 76 – 84 peptide, 100 µl of the sample plasma from patient #12 was cultured in plates which were pre-coated with the indicated peptides. Thereafter, the levels of IgG reactive to the PSCA 76 – 84 peptide in the resultant samples were determined by enzyme-linked immunosorbent assay (ELISA). Values represent the mean of triplicate assays. *Statistically significant value at $P < 0.05$.

PSCA has been reported to be a target candidate of humoral immunity. The anti-tumor effect of anti-PSCA mAb was revealed in a study by using human prostate cancer xenograft mouse models and using androgen-dependent and androgen-independent cell lines [32]. The administration of anti-PSCA mAb inhibited the formation of subcutaneously inoculated or orthotopic xenograft tumors. In addition, the administration of anti-PSCA mAbs led to the retardation of established orthotopic tumor growth, and the inhibition of metastasis to distant sites. Furthermore, human PSCA-expressing established xenograft tumors were completely regressed in a large proportion of animals by the administration of anti-PSCA mAb conjugated with maytansinoid [33]. These results suggest that PSCA is an attractive target for immunotherapy against prostate cancer not only with respect to cellular immunity but also to humoral immunity.

In conclusion, we identified a new PSCA-derived peptide immunogenic in HLA-A24$^+$ prostate cancer patients. The frequencies of the HLA-A24 allele are relatively high throughout the world [34]. This information could extend the possibility of treating HLA-A24$^+$ patients with HRPC and/or metastases using peptide-based specific immunotherapy.

ACKNOWLEDGMENTS

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

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Report

Identification of HER2/*neu*-derived peptides capable of inducing both cellular and humoral immune responses in HLA-A24 positive breast cancer patients

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Key words: Ab, cancer vaccine, CTL, HER2/*neu*, peptides

Summary

HRE2/*neu*-specific cellular and humoral immune responses are often detected in breast cancer patients, but identification of more immunogenic CTL epitope peptides is necessary prior to development of a cancer vaccine. There is accumulating evidence of strong immunogenicity of peptides capable of inducing both cellular and humoral immune responses. To identify such peptides, this study intended to determine HER2/*neu*-derived peptides capable of inducing both cellular and humoral immunity in HLA-A24$^+$ breast cancer patients. IgGs reactive to the $HER2_{342-350}$, $HER2_{485-493}$, and $HER2_{553-561}$ peptides were detected in the sera of these patients with the frequency of 47, 24, and 24%, respectively. These peptides also induced peptide-specific and tumor-reactive CTL activity in the peripheral blood mononuclear cells of HLA-A24$^+$ breast cancer patients with the frequency of 50, 63, and 25%, respectively, but such activity was not induced from any HLA-A24$^-$ patients. Cellular and humoral responses to each of these three peptides were also observed in PBMCs and sera from the other epithelial cancer patients. These results may provide a scientific basis for new clinical trials of HER2/*neu*-peptide-based immunotherapy for breast cancer and also other epithelial cancer patients.

Abbreviations: aa: amino acid; ELISA: enzyme-linked immuno-sorbent assay; E/T, effector to target; IL: interleukin; nt: nucleotide; PBMC: peripheral blood mononuclear cells; PHA: phytohemagglutinin

Introduction

HER2/*neu* is a 185-kD trans-membrane glycoprotein that has tyrosine kinase activity [1, 2]. Overexpression of HER2/*neu* is observed in 30–40% of breast and ovarian cancers in association with poor prognosis [3, 4]. Cellular and humoral immune responses to HER2/*neu* antigen were found to be detectable in substantial proportions of breast and ovarian cancer patients [5, 6]. Administration of herceptin, a humanized anti-HER2/*neu* mAb, resulted in tumor regression in breast cancer patients whose tumors overexpressed the antigen [4, 7]. In view of development of peptide-based cancer therapy, some immunogenic HER2/*neu*-peptides capable of inducing HLA-A allele (-A2, -A3, -A24)-restricted CTLs have been reported in the past decade [6, 8–11]. However, as far as we know, these peptides induced CTLs failed to recognize tumor cells *in vivo* in the initial clinical trials [12]. This failure could indicate weak immunogenicity of these peptides. Notably, there is a line of evidence suggesting strong immunogenicity of peptides capable of inducing both cellular and humoral immune responses [13–15]. We previously reported that some CTL-directed peptides demonstrated the ability to elicit both cellular and humoral immune responses in clinical studies [16, 17]. Further, levels of anti-peptide Ab in post-vaccination sera were well correlated with the overall survival of advanced lung cancer patients who received peptide vaccination [17]. Therefore, HER2/*neu*-peptides capable of inducing both cellular and humoral responses may be more im-

munogenic than those of cellular response alone. We have attempted in this study to identify such peptides, and report the three peptides that may be considered as new vaccine candidates for HLA-A24$^+$ patients with breast cancer and other epithelial cancers.

Materials and methods

Samples and cell lines. After informed consent had been obtained, sera, peripheral blood mononuclear cells (PBMCs), and tissues were collected from 17 patients diagnosed with breast cancer at Kurume University Hospital. Expression of HER2/*neu* antigen in these breast cancer tissues were investigated by two pathologists at Kurume University Hospital using an immuno-histochemistry test (HercepTest^{TM}, Japan Roche, Tokyo) [18]. In this study, cancer tissues with scores of 0 or 1+ were judged as negative for HER2/*neu*, and those with a score of 2 or 3 positive for HER2/*neu*. PBMCs and sera were also obtained from eight female healthy donors (HDs). After informed consent had been obtained, sera and PBMCs were also collected from 30 cancer patients (14 patients with prostate cancer, 8 colon cancer, 3 cervical cancer, 3 gastric cancer, 2 breast cancer) to study their responses to HER2/*neu*-derived peptides. All sera were stored at −80°C, while PBMCs were cryopreserved at −196°C until use. HER2/*neu*-expressing human ovarian cancer cell line SKOV3 (HLA-A3/28) and its HLA-A24-transfected SKOV3-A24 were kindly provided by Dr Hiroshi Shiku (Mie University, Mie, Japan) [10]. C1R-A2402 (an HLA-2402 transfectant cell line) was kindly provided by Dr Masafumi Takiguchi (Kumamoto University, Kumamoto, Japan). Phytohemagglutinin (PHA)-blastoid T cells from PBMCs were used as a negative control of target cells for a ^{51}Cr-release assay.

Peptides and quantification of peptide-specific IgG. Peptides were purchased from BioSynthesis (Lewisville, TX). Among them, HER2$_{8-16}$ and HER2$_{63-71}$ had been reported as CTL peptides recognized by the HLA-A24-restricted CTLs [10, 11]. Because no subject in this study was a carrier of HIV, an HIV peptide (RYLRDQQLL) with HLA-A24 binding motif was used as a negative control. Peptide-specific IgG levels in sera were measured by enzyme-linked immunosorbent assay (ELISA) as reported previously [19]. In brief, serum samples were serially diluted with 0.05% Tween 20-Block Ace (MEGMILK, Hokkaido, Japan), and 100 μl/well of diluted serum were added to the peptide (20 μg/well)-immobilized Nunc Covalink plates (Roskilde, Denmark). Antibodies (Ab) were detected with rabbit anti-human IgG (γ-chain-specific) (DAKO, Glostrup, Denmark). For determining the sensitivity limit of the ELISA, sera from 14 healthy (HIV-negative) donors were measured for their reactivity to HIV peptide, taken as a negative control peptide, by the assays. The mean ± SD of optical density by ELISA was indicated at 0.040 ± 0.030. The mean ± SD value (0.070) was determined as the cut-off value. To test the specificity of anti-peptide activity in each serum sample, 100 μl/well of serum samples (×100 dilution with 0.05% Tween 20-Block Ace) were absorbed with immobilized peptides (20 μg/well) in wells of the plate for 2 h at 37°C. The absorption was repeated three times followed by testing of the activity with ELISA.

CTL induction. For induction of peptide-specific CTLs, PBMCs (1×10^5 cells/well) were incubated with 10 μM of each peptide in the wells of a 96-well U-bottom microculture plate (Nunc) in 200 μl culture medium containing IL-2, as reported previously [16]. On the 14th day of culture, the cells were harvested, washed, and tested for their ability to produce IFN-γ in response to C1R-A24 pulsed with a corresponding peptide or a negative control peptide (HIV). After an 18-h incubation, the supernatant was collected for measurement of IFN-γ by ELISA. The PBMCs showing a positive response were collected and further cultured with IL-2 alone for 10–14 days for a standard 6 h ^{51}Cr-release assay [16]. For the inhibition test, 20 μg/ml of anti-HLA-class I (W6/32, IgG2a), anti-HLA-A2 (BB7.2, IgG2b), anti-CD8 (Nu-Ts/c, IgG2a), anti-HLA-class II (H-DR-1, IgG2a), and anti-CD4 (Nu-Th/i, IgG1) mAb were used. Anti-CD14 (JML-H14, IgG2a) mAb served as a control. A two-tailed Student's *t*-test was employed for the statistical analysis.

Results

*Identification of IgG reactive HER2/*neu *peptides.* We first investigated whether IgG reactive to HER2/*neu*-derived peptides with HLA-A24 binding motif could be detected in sera of 17 breast cancer patients (13 patients with HER2/*neu*-positive tumors and 4 patients with HER2/*neu*-negative tumors) and eight female HDs. Significant levels of IgG react-

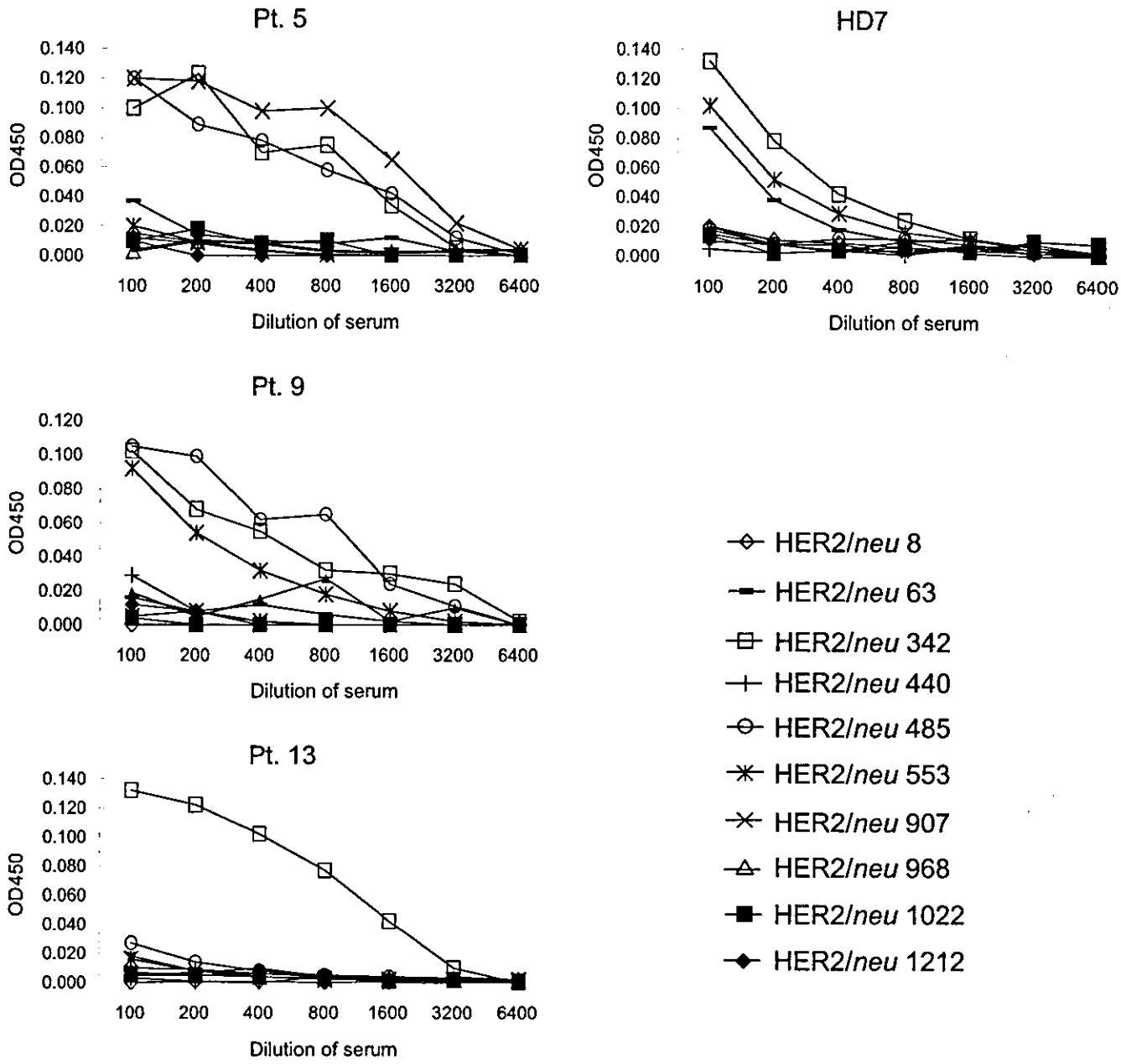

Figure 1. Detection of anti-HER2/*neu* peptide IgG. Optical density (OD) values of each sample were assayed in serially diluted samples to estimate peptide-specific IgG levels. The OD value against an irrelevant peptide (HIV) used as a negative control was subtracted from the data. The times-dilution of sera is shown on the horizontal axis. Representative results of Pts. 5, 9, and 13, and HD7 are shown.

ive to the $HER2_{342-350}$, $HER2_{485-493}$, $HER2_{553-561}$, $HER2_{907-915}$, and $HER2_{968-976}$ peptides were detected in the sera of 8, 4, 4, 3, and 1 HLA-A24$^+$ breast cancer patient, respectively. In all cases except one (Pt. 10) whose tumor was negative of HER2/*neu*, these positive sera were from patients with HER2/*neu*-positive tumor by an immuno-hisotochemistry test (data not shown). In contrast, significant levels of IgG reactive to the remaining four peptides, two of which had been reported to be recognized by the HLA-A24-restricted CTLs [10, 11], were entirely undetectable. Sera from 4, 1 and 1 of the 8 HDs tested also showed the significant levels of IgG reactive to $HER2_{342-350}$, $HER2_{63-71}$, and $HER2_{553-561}$, respectively. Representative results of the four cases (Pts. 5, 9, and 13, and HD7) are shown in Figure 1, and a summary of the results is given in Table 1. The peptide specificity of anti-peptide IgG, except in the case of anti-$HER2_{553-561}$IgG, was confirmed by absorption test. Representative results are shown in Figure 2. In contrast, anti-$HER2_{553-561}$ activity was also absorbed with irrelevant peptides, including an HIV peptide used as a negative control, but it was not absorbed with the HIV peptide (PFRDYVDRF) with

Table 1. Humoral and cellular responses to the HER2/*neu* peptides

	HER2/*neu* over-expression	HLA	Responses to the HER2/*neu* peptides (data were shown as IFN-γ production (pg/ml)[a]/OD at 450 nm in									
			HER2/*neu* 8	HER2/*neu* 63	HER2/*neu* 342	HER2/*neu* 440	HER2/*neu* 485	HER2/*neu* 553	HER2/*neu* 907	HER2/*neu* 968	HER2/*neu* 1022	HER2/*neu* 1212
Pt. 2	HER2(+)	A33	[c]/–[d]	/–	/–	/–	/0.107	/0.101	0/0.088	/–	/–	/–
Pt. 4	HER2(+)	A24	0/–	156/–	0/0.093	0/–	102/–	0/–	0/–	0/0.140	0/–	0/–
Pt. 5	HER2(+)	A11/24	0/–	0/–	0/0.137	0/–	0/0.120	0/–	0/0.120	0/–	0/–	0/–
Pt. 6	HER2(+)	A2/24	0/–	0/–	103/0.106	0/–	0/–	170/–	160/–	0/–	0/–	0/–
Pt. 7	HER2(+)	A11/33	/–	/–	/0.095	/–	/–	/–	/–	/–	/–	/–
Pt. 8	HER2(+)	[c]	/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
Pt. 9	HER2(+)	A1/24	0/–	0/–	158/0.102	0/–	100/0.105	207/0.092	0/–	0/–	0/–	0/–
Pt. 12	HER2(+)		/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
Pt. 13	HER2(+)	A24	107/–	0/–	133/0.132	0/–	179/–	0/–	0/–	0/–	0/–	0/–
Pt. 14	HER2(+)	A2/31	/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
Pt. 15	HER2(+)	A33	/–	/–	/–	/–	/–	/0.146	/–	/–	/–	/–
Pt. 16	HER2(+)	A33/24	0/–	0/–	0/0.116	0/–	128/–	0/–	0/–	0/–	0/–	0/–
Pt. 17	HER2(+)	A2/24	/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
Pt. 1	HER2(–)	A26/31	/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
Pt. 3	HER2(–)	A2/26	/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
Pt. 10	HER2(–)	A3/24	0/–	100/–	122/0.146	0/–	132/0.339	0/0.121	0/0.156	0/–	0/–	0/–
Pt. 11	HER2(–)	A24	0/–	0/–	0/–	0/–	0/–	0/–	0/–	0/–	0/–	0/–
HD1	[c]		/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
HD2		A2/24	157/–	0/–	0/0.141	0/–	0/–	0/–	0/–	0/–	0/–	0/–
HD3		A2/24	0/–	0/–	0/0.092	0/–	0/–	0/–	0/–	0/–	0/–	0/–
HD4			/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
HD5		A24/11	0/–	533/–	622/0.414	0/–	0/–	0/–	0/–	0/–	0/–	0/–
HD6			/–	/–	/–	/–	/–	/–	/–	/–	/–	/–
HD7		A24/11	0/–	163/0.087	124/0.136	0/–	102/-	0/0.106	0/–	0/–	0/–	0/–
HD8		A24	0/–	0/–	0/–	0/–	0/–	0/–	0/–	0/–	0/–	0/–
CTL induction		Pt. ($n=8$)	1	2	4	0	5	2	1	0	0	0
		HD ($n=5$)	1	2	2	0	1	0	0	0	0	0
Anti-peptides Abs		Pt. ($n=17$)	0	0	8	0	4	4	3	1	0	0
		HD ($n=8$)	0	1	4	0	0	1	0	0	0	0

[a] PBMCs from HLA-A24+ breast cancer patients were *in vitro* stimulated with indicated HER2 peptides, as described in Materials and methods section. On day 14, the cultured PBMCs were tested for their reactivity to C1R-A24, which were pre-pulsed with a corresponding peptide in quadruplicate. Values represent the result of the best of the four wells, and background IFN-γ production in response to the HIV peptide (taken as a negative control) was subtracted. Successful induction of peptide-specific CTLs was judged to be positive when more than 100 pg/ml IFN-γ production was observed in response to corresponding peptide-pulsed CIR-A24 cells compared to HIV peptide-pulsed CIR-A24 cells.

[b] Anti-peptide IgG was assayed by ELISA as described in Materials and methods section.

[c] (blank): not determined.

[d] The OD values lower than the cut-off (0.070) were shown as –.

Table 2. Humoral and cellular responses to the HER2/*neu* peptides in HLA-A24$^+$ epithelial cancer patients[a]

Cancer[b]	Responses to the HER2/*neu* peptides (data were shown as IFN-γ production (pg/ml)/OD at 450 nm		
	HER2/*neu* 342	HER2/*neu* 485	HER2/*neu* 553
PC1	/0.70	/–	/1.41
PC2	/–	/0.07	/0.15
PC3	/0.70	/0.07	/0.15
PC4	/–	/–	/0.07
PC5	/–	/–	/0.13
PC6	101/0.07	–/–	–/–
PC7	/0.07	/0.08	/–
PC8	/0.07	/–	/0.01
PC9	–/–	–/–	103/–
PC10	–/–	–/–	–/0.07
PC11	110/–	122/–	–/0.11
PC12–14	/–	/–	/–
CC1	–/–	107/–	112/0.12
CC2	104/–	–/–	106/0.10
CC3	–/–	–/–	106/0.19
CC4	172/0.17	–/0.12	138/0.25
CC5	–/0.08	101/0.12	–/0.12
CC6	–/0.11	–/–	–/0.16
CC7	–/–	–/–	–/0.06
CC8	–/–	–/–	–/–
UCC1	–/0.08	109/0.07	112/–
UCC2	–/–	–/–	–/0.10
UCC3	–/–	–/–	–/0.08
GC1	282/–	837/–	106/–
GC2	–/–	–/–	–/0.08
GC3	101/–	–/–	–/–
BC1	–/–	–/–	–/0.12
BC2	–/–	122/–	–/–
CTL induction ($n = 20$)	6	6	7
Anti-peptide Ab ($n = 30$)	9	6	19

[a] Please see the footnote of Table 1.
[b] PC: prostate cancer, CC: colon cancer, UCC: uterine cervical cancer, GC: gastric cancer, BC: breast cancer.

HLA-B4601 binding motif [20], suggesting its cross-reactivity to the other peptides (data not shown). Based on these findings, the $HER2_{342–350}$, $HER2_{485–493}$, $HER2_{553–561}$, and $HER2_{907–915}$ peptides, along with two previously reported CTL epitopes ($HER2_{8–16}$ and $HER2_{63–71}$), were tested for their ability to induce CTL activity in the following experiments.

Identification of peptides capable of inducing cellular immunity. PBMCs from eight breast cancer patients (six patients with HER2/*neu*-positive tumors and two patients with HER2/*neu*-negative tumors) and five female HDs were incubated with each of six kinds of peptides (>90% purity) or a control HIV peptide, and subsequently examined for their IFN-γ production in response to corresponding peptide-pulsed C1R-A24 cells (Table 1). The previously reported $HER2_{8–16}$ and $HER2_{63–71}$ peptides induced peptide-specific IFN-γ production in one and two patients, respectively. The $HER2_{342–350}$, $HER2_{485–493}$, $HER2_{553–561}$, and $HER2_{907–915}$ peptides induced peptide-specific IFN-γ production in four, five, two, and one patients,

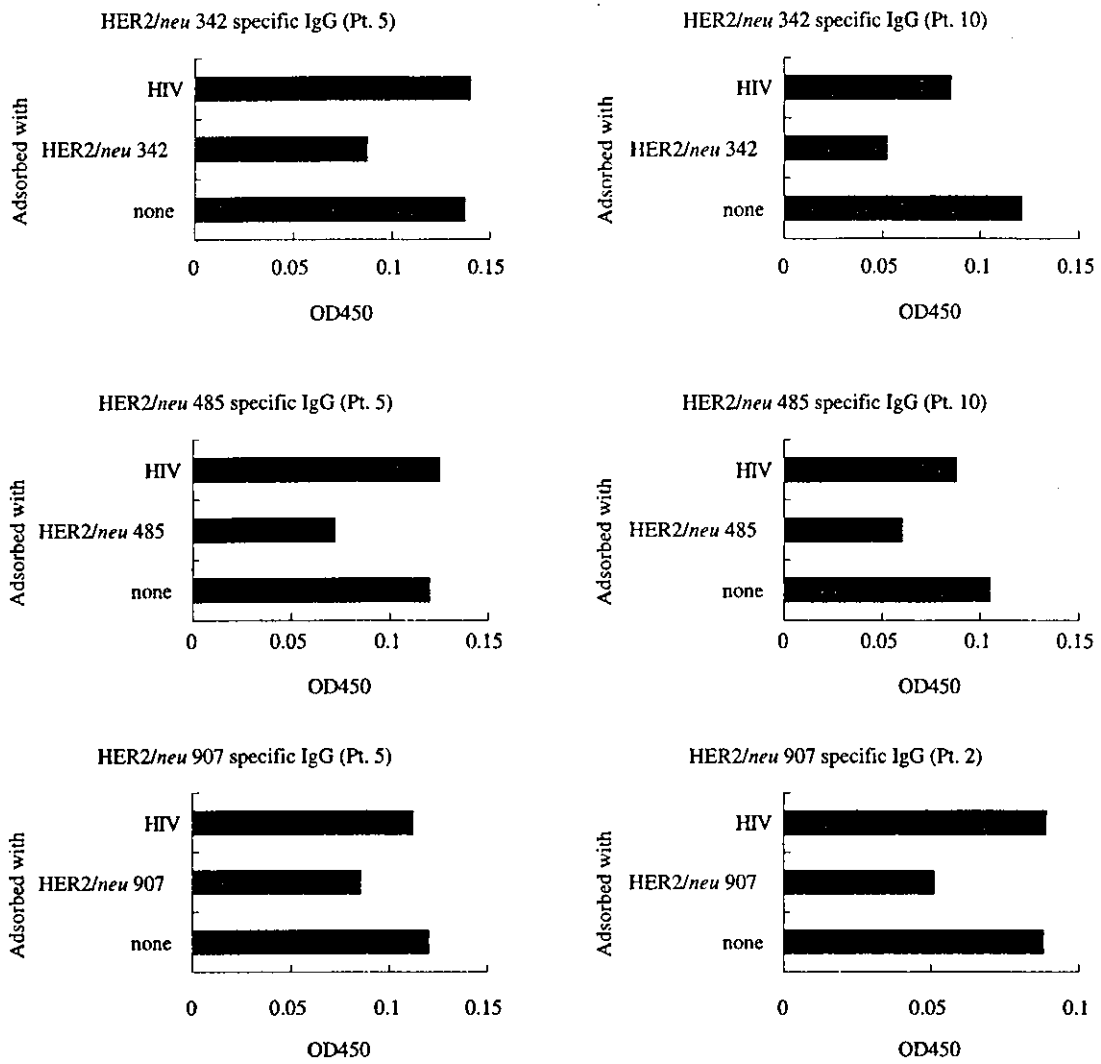

Figure 2. Specificity of anti-peptide IgG. Sera samples were absorbed with immobilized peptides three times at 37°C followed by testing of peptide-specific IgG with ELISA. The representative results in Pts. 2, 5 and 10 are shown in this figure. The concentrations of IgG in sera are shown as OD at 450 nm. Values represent the means of duplicate assays.

respectively. Some of these peptides also induced such activity in a few HDs. Levels of IFN-γ produced by these PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by other mAb in the assay, suggesting that these CTL activities were largely mediated by $CD8^+$ T cells in an HLA-HLA-class I-restricted manner (data not shown). In contrast to HLA-$A24^+$ PBMCs, HLA-$A24^-$ PBMCs from any of eight breast cancer patients in Table 1 produced IFN-γ production in response to HER2/*neu* peptides shown above (data not shown). These results indicate that IFN-γ production in response to each of the $HER2_{342-350}$, $HER2_{485-493}$, $HER2_{553-561}$, and $HER2_{907-915}$ peptides was mediated by $CD8^+$ T cells with an HLA-A24-restricted manner.

Further, these PBMCs showing a positive response displayed significant levels of cytotoxicity against the SKOV3-A24 cells (HLA-$A24^+$, HER2/neu^+), but failed to lyse SKOV3 cells (HLA-$A24^-$, $HER2^+$) or PHA-blastoid T cells (HLA-$A24^+$) in all the cases tested. Representative results of the six cases (HD7, Pts. 6, 9, 10, 13, and 16) are shown in Figure 3(A). The peptide-stimulated PBMCs in all the cases tested showed relatively higher levels of cytotoxicity against the SKOV3-A24 cells, but no or very low levels of the cytotoxicity to SKOV3 cells (Figure 3(A)) or PHA-blastoid T cells (data not shown). PBMCs stimulated with an HIV peptide, taken as a negative control, did not show such cytotoxicity (Figure 3(A)). HLA-class I restriction of the cytotoxicity was then confirmed by inhibition test with anti-HLA-class I mAb. Namely, the cytotoxicity against SKOV3-A24 tumor cells by the peptide-stimulated PBMCs was significantly inhibited by anti-HLA-class-1 mAb in all the cases tested (Figure 3(B)). All these results suggest

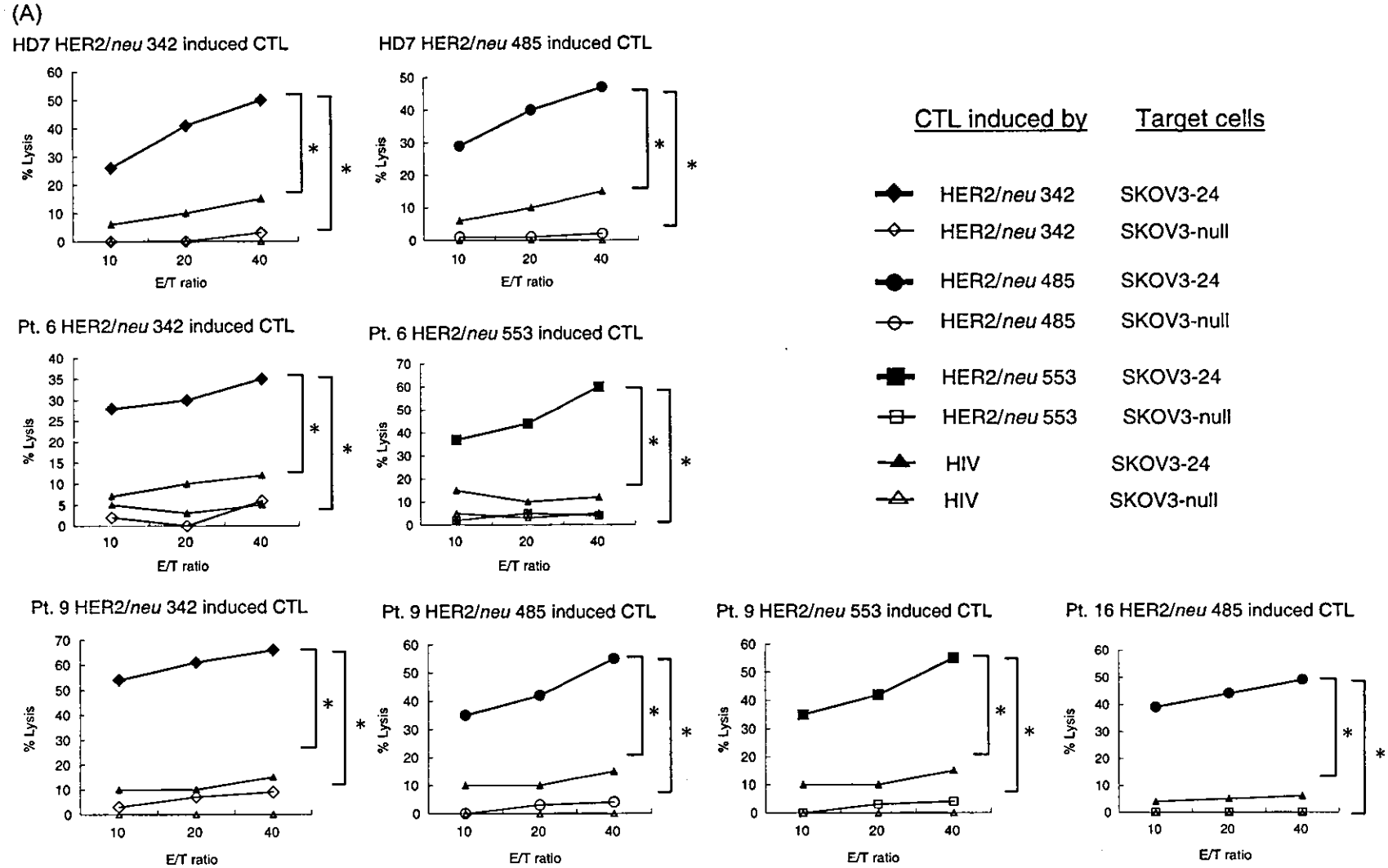

Figure 3. Cytotoxicity. (A) Peptide-stimulated PBMCs were tested for their cytotoxicity to HLA-A24-transfected and HER2/*neu*-expressing ovarian cancer cell line SKOV3 and HLA-A24 untransfected SKOV3 (SKOV3-null) cells, and PHA-blastoid normal T cells established from HLA-A24$^+$ HDs by 6-h ^{51}Cr-release assay at three different E/T ratios. Representative data from HD7, Pts. 6, 9, and 16 are shown. The percent cytotoxicity against HLA-A24+ PHA-blastoid T cells was <3% in all the cases tested, and thus the data were not shown in the figure. Values represent the means of triplicate assays. A two-tailed Student's *t*-test was used for statistical analysis of the percentage of cell lysis between to SCOV3-null and SKOV3-24, or SCOV3-24 and SKOV3-24 with anti-class I mAb. $^*P < 0.05$. (B) Peptide-stimulated PBMCs were also tested for their cytotoxicity to HLA-A24-transfected and HER2/*neu*-expressing ovarian cancer cell line SKOV3 and HLA-A24 untransfected SKOV3 (SKOV3-null) cells by 6-h ^{51}Cr-release assay at three different E/T.ratios in the absence or presence of 20 μg/ml of anti-HLA-class I (W6/32) mAb. Representative data from Pts. 9, 10 and 13 are shown. Values represent the means of triplicate assays. A two-tailed Student's *t*-test was used for statistical analysis of the percentage of cell lysis between to SCOV3-null and SKOV3-24, or SCOV3-24 and SKOV3-24 with anti-class I mAb. $^*P < 0.05$.

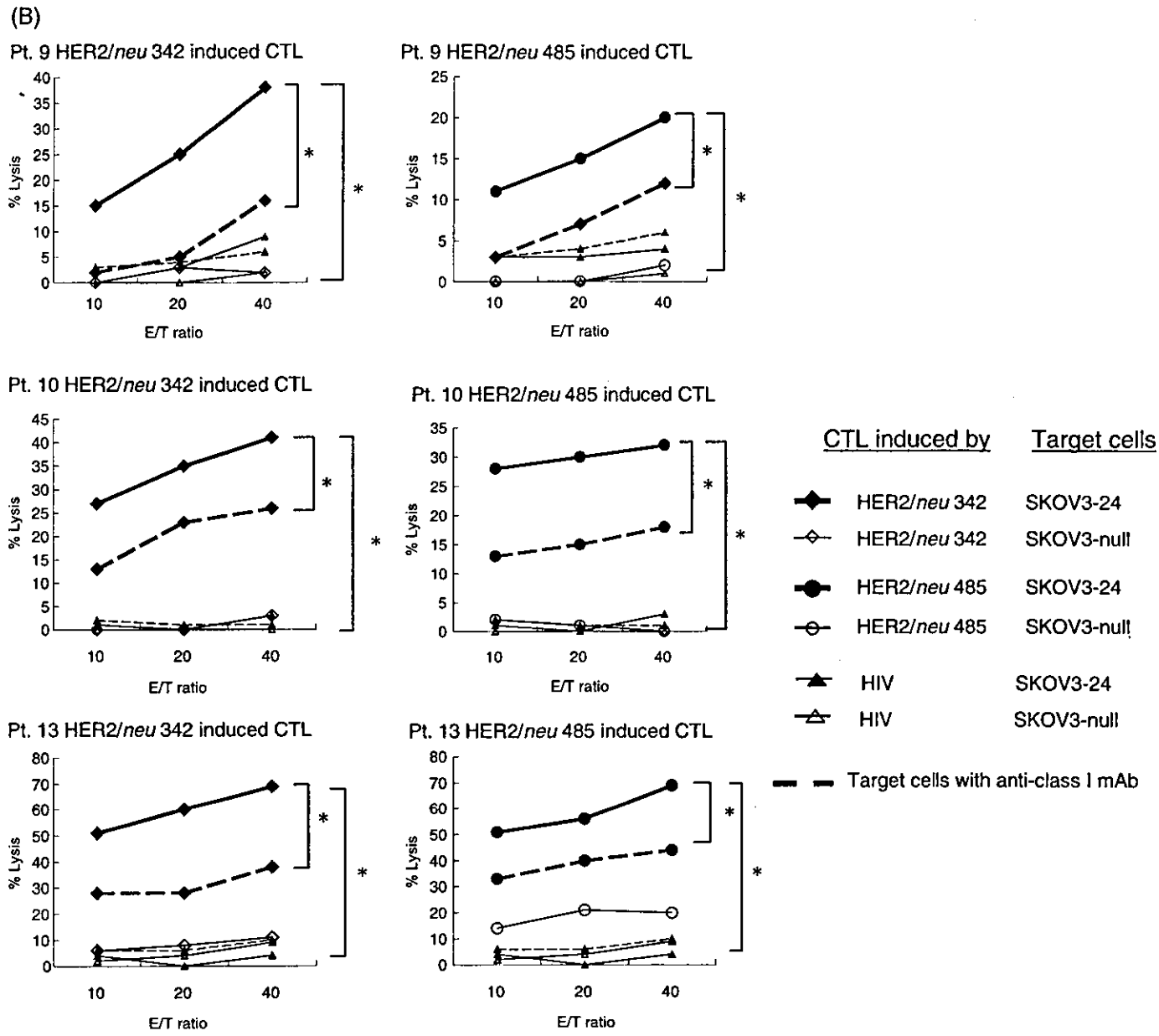

Figure 3. continued.

that these CTL activities were restricted to HLA-A24 molecules.

Responses in the other epithelial cancer patients. In order to study responses of the other epithelial cancer patients to HER2/*neu* peptides, sera and/or PBMCs from 30 epithelial cancer patients (14 patients with prostate cancer, 8 colon cancer, 3 cervical cancer, 3 gastric cancer, and 2 breast cancer) were tested for their responses to three different HER2/*neu*-derived peptides to which immune responses were observed with relatively higher frequencies (Table 1). The $HER2_{342-350}$, $HER2_{485-493}$, and $HER2_{553-561}$ peptides induced peptide-specific IFN-γ production in 6, 6 and 7 patients among 20 patients tested, respectively. Significant levels of IgG reactive to the $HER2_{342-350}$, $HER2_{485-493}$, and $HER2_{553-561}$ peptides were detected in sera from 9, 6, and 19 among 30 patients tested, respectively. These results indicate that cellular and humoral responses to each of the three HER2/*neu* peptides were observed in various types of epithelial cancer patients. It is of note that IgG reactive to $HER2_{342-350}$ or $HER2_{553-561}$ peptide was most frequently observed in sera of breast cancer patients (8 of 19 cases, 42%) or those of epithelial cancer patients other than breast cancer patients (19 of 28 cases, 63%).

Discussion

This study reported the three HER2/*neu*-derived peptides at positions 342–350, 485–493, and 553–561 were recognized by both sera (IgG) and PBMCs from the substantial numbers of breast and the other epithelial cancer patients, respectively. In contrast, the previously reported $HER2_{8-16}$ and $HER2_{63-71}$ peptides [10, 11], used as reference peptides in this study, rarely induced peptide-specific IFN-γ production. This discrepancy might be explained by the fact that PBMCs from breast cancer patients were provided for this study, while those from ovarian [10] and colorectal cancer patients [11] were provided in the previous studies. This study also showed that IgG reactive to the reference peptides were not detected at all in sera of breast cancer patients, although there was no reports of humoral responses to any of the HER2/*neu*-derived peptides with HLA-class I binding motifs in the previous studies [10, 11].

Both cellular and humoral responses to $HER2_{342-350}$, $HER2_{485-493}$, and $HER2_{553-561}$ peptides were not only observed in PBMCs from breast cancer patients but also those of prostate and colon cancer patients. This phenomenon is expected primarily because of HER2/*neu* expression in various types of epithelial cancers [1–4]. The HLA-A24 allele was found in 60% of Japanese (with 95% of these cases being genotypically A2402), 20% of Caucasians, and 12% of Africans [21].

All the results of the present studies suggest the potential use of $HER2_{342-350}$, $HER2_{485-493}$, and $HER2_{553-561}$ peptides for clinical trials as cancer vaccine for HLA-$A24^+$ epithelial cancer patients. Subsequently, the three peptides are currently used as peptide vaccines for HLA-$A24^+$ breast, prostate, gastric, and colorectal cancer patients in Kurume and Hokkaido University Hospitals under an individualized vaccination regimen as reported previously [16, 17, 22, 23]. Although the clinical study is on going and the results shall be reported in future as a separate manuscript, four patients (three colon and one gastric cancer patients) were vaccinated with $HER2_{553-561}$ peptides, and one patient received $HER2_{553-561}$ peptide. Local skin reactions at the injection sites were observed in two patients, but no other adverse events were associated with the vaccination. Post (6th)-vaccination sera and PBMCs from 4 and 2 of 5 patients showed increased levels of humoral and cellular responses to the vaccinated peptides, respectively. Further clinical and basic studies are needed to show the immunogenecity of these three peptides *in vivo* from a point of anti-tumor responses.

We previously reported that IgGs reactive against CTL epitope peptides were often detected in the pre-vaccination sera of cancer patients and also in the sera of HDs [16, 17, 19]. Further, some CTL-directed peptides have been reported in clinical studies to be able to elicit both cellular and humoral immune responses, and levels of anti-peptide Abs in post-vaccination sera well correlated with overall survival of advanced lung cancer patients who received peptide vaccination [17]. In contrast, IgGs reactive to these peptides are either absent or unbalanced in the sera of patients with atopic disease [19]. These results suggest that IgG reacting to these peptides plays a role in host-defense against various diseases, although the underlying mechanism of anti-tumor immune responses in cancer patients is presently unclear. The peptide-IgGs tested did not react to the mother proteins, and also failed to show either direct growth inhibition of tumor cells in vitro or to elicit antibody-dependent cell-mediated cytotoxicity to tumor cells (data not shown). Therefore, the anti-HER/*neu* peptide-IgG shown in this study may not play a role in direct action to tumor cells. Rather, these Abs may be involved in infiltration of immunocompetent cells into tumor sites through induction of inflammatory reactions around these sites (Noguchi et al., unpublished results).

IgG reactive to HER2/*neu* peptides were found in sera of both cancer patients and HDs, and also in sera of both HLA-$A24^+$ and $-A24^-$ subjects. IgG reactive to these peptides were also observed in sera of both patients whose tumors over expressed HER2/*neu* antigen and those not expressed it. The existence of peptide-specific IgG in sera of HDs and patients with atopic disease of IgG reactive was previously reported [16, 17, 19]. Further, immune responses of both cancer patients and HDs to HER2/*neu* peptides may not surprise us since it is expressed not only in epithelial cancer cells but also in certain normal epithelial cells. Indeed, CTL precursors for peptides of HER2/*neu* are also detectable in PBMCs from both cancer patients and HDs [10, 11]. On the contrary, mechanisms of no apparent HLA-class I restriction on the presence of peptide-specific IgG, are not clear at the present time, although its existence in sera of HDs were also observed in IgG reactive to other CTL epitope peptides as reported previously [16, 17, 19]. Further studies are needed to clarify the biological role as well as the mechanism of no HLA-class I restriction of IgG reactive to CTL epitope peptides.

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Identification of parathyroid hormone-related protein-derived peptides immunogenic in human histocompatibility leukocyte antigen-A24$^+$ prostate cancer patients

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Parathyroid hormone-related protein (PTHrP) is a key factor in the development of bone metastases, which are a major barrier in treating prostate cancer patients. In this study, we attempted to identify PTHrP-derived peptides immunogenic in human histocompatibility leukocyte antigen (HLA)-A24$^+$ prostate cancer patients. Among four different PTHrP peptides carrying the HLA-A24 binding motif, both the $PTHrP_{36-44}$ and $PTHrP_{102-111}$ peptides efficiently induced peptide-specific cytotoxic T lymphocytes from peripheral blood mononuclear cells (PBMCs) of HLA-A24$^+$ prostate cancer patients. Peptide-stimulated PBMCs showed cytotoxicity against prostate cancer cells in an HLA-A24-restricted manner. Experiments using antibodies and cold inhibition targets confirmed that their cytotoxicity was dependent on PTHrP peptide-specific and CD8$^+$ T cells. Immunoglobulin G reactive to the $PTHrP_{102-111}$ or $PTHrP_{110-119}$ peptide was frequently detected in the plasma of prostate cancer patients, suggesting that the $PTHrP_{102-111}$ peptide is able to elicit cellular and humoral immune responses in cancer patients. These results indicate that the PTHrP could be a promising target molecule for specific immunotherapy of HLA-A24$^+$ prostate cancer patients with metastases.
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Prostate cancer is one of the most common cancers among elderly men (Greenlee *et al*, 2000). Prostate cancer frequently metastasises to bone. Androgen withdrawal therapy has been applied for patients with bone metastases. Although hormone therapy can temporarily inhibit the progress of the disease in these patients, a progression to hormone-refractory prostate cancer inevitably occurs in most cases. Therefore, the development of new therapeutic modalities is needed.

Recent advances in tumour immunology have allowed us to identify the genes encoding human cancer-related antigens, and the epitopes, which are recognized by cytotoxic T lymphocytes (CTLs), in patients with various types of cancers (Boon *et al*, 1997; Rosenberg, 1999; Renkvist *et al*, 2001). The identified tumour antigens and their peptides have been applied for specific immunotherapy (Nestle *et al*, 1998; Rosenberg *et al*, 1998; Marchand *et al*, 1999). In the case of prostate cancer, tissue-specific antigens, which are expressed in the normal prostate, can also be target molecules for specific immunotherapy for patients with this disease. Immunotherapy targeting prostate-specific antigens or prostate-specific membrane antigens has been carried out, and antitumour effects have been observed in limited cases (Murphy *et al*, 1996, 1999; Tjoa *et al*, 1998; Small *et al*, 2000; Gulley *et al*, 2002).

Parathyroid hormone-related protein (PTHrP) is an autocrine or paracrine factor that binds to receptors on osteoblasts, and stimulates bone formation and reabsorption. Parathyroid hormone-related protein has limited homology with PTH at its NH2 terminus, and can bind to the same receptor as PTH, resulting in similar biological activity (Suva *et al*, 1987; Juppner *et al*, 1991). Parathyroid hormone-related protein plays a variety of physiological roles, including calcium transport, keratinocyte differentiation, smooth muscle relaxation, and cartilage development (Philbrick *et al*, 1996). In parathyroid cells, a high extracellular calcium concentration inhibits parathyroid hormone (PTH) secretion and the proliferation of parathyroid cells as a result of negative feedback regulation, whereas it evokes further PTHrP secretion and promotes worsening bone resorption (Sanders *et al*, 2001). Therefore, PTHrP has been considered to be responsible for the hypercalcemia associated with malignancy (Guise, 1997). In addition, prostate cancers have been reported to produce PTHrP (Francini *et al*, 2002). These lines of evidence suggest that PTHrP could be a promising target molecule for the immunotherapy of prostate cancer patients with bone metastases. In this study, we

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attempted to identify new, PTHrP-derived peptides that are immunogenic in HLA-A24$^+$ prostate cancer patients.

MATERIALS AND METHODS

Patients

Informed consent was obtained from all of the HLA-A24$^+$ prostate cancer patients and HLA-A24$^+$ healthy volunteers who were enrolled in this study. None of the participants were infected with HIV. In total, 20 ml of peripheral blood was obtained, and the PBMCs were prepared by Ficoll-Conray density gradient centrifugation. The expression of HLA-A24 molecules on the PBMCs of the cancer patients and healthy donors was determined by flow cytometry.

Cell lines

C1R-A24 is an HLA-A*2402-expressing subline of C1R lymphoma (Dr M Takiguchi, Kumamoto University, Japan). LNCaP is an HLA-A24 negative prostate cancer cell line. To establish LNCaP cells that stably express HLA-A24 molecules (designated as LNCaP-A24), an *HLA-A*2402* gene was inserted into a pcDNA3.1/Hygro vector (Invitrogen, CA, USA), and electroporated into the LNCaP cell line (ATCC, Manassas, VA, USA), and selection was carried out with hygromycin B (Invitrogen) at a dose of 170 $\mu g\,ml^{-1}$. All cell lines were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FCS.

Peptides

Four PTHrP-derived peptides (listed in Table 1) were prepared based on the HLA-A24 binding motif (Parker *et al*, 1994; Rammensee *et al*, 1995). All peptides were of >90% purity and were purchased from Biologica Co., Nagoya, Japan. Influenza (Flu) virus-derived (RFYIQMCYEL), EBV-derived (TYGPVFMCL), and HIV-derived peptides (RYLRQQLLGI) with the HLA-A24 binding motif were used as controls. All peptides were dissolved with DMSO at a dose of 10 $mg\,ml^{-1}$.

Assay for peptide-specific CTLs in PBMCs

The assay for the detection of peptide-specific CTLs in PBMCs was performed according to a previously reported method (Hida *et al*, 2002). In brief, PBMCs (1×10^5 cells per well) were incubated with 10 $\mu g\,ml^{-1}$ of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) at a volume of 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Gibco BRL), 10% FCS, 100 $U\,ml^{-1}$ of IL-2, and 0.1 mM MEM nonessential amino-acid solution (Gibco, BRL). Half of the culture medium was removed and replaced with new medium containing a corresponding peptide (20 $\mu g\,ml^{-1}$) every 3 days. On the 15th day of culture, the cultured cells were separated into four wells; two wells were used for the PTHrP peptide-pulsed C1R-A24 cells, and the other two wells were used for the HIV peptide-pulsed C1R-A24 cells. After an 18-h incubation period, the supernatants were collected, and the level of IFN-γ was determined by ELISA (limit of sensitivity: 10 $pg\,ml^{-1}$).

Table 1 Reactivity of PTHrP peptide-stimulated PBMCs from HLA-A24$^+$ healthy donors and prostate cancer patients

	Peptides					
	Name	$PTHrP_{36-44}$	$PTHrP_{102-111}$	$PTHrP_{25-34}$	$PTHrP_{110-119}$	Flu	EBV
PBMCs	Amino-acid sequence	RAVSEHQLL	RYLTQETNKV	RSVEGLSRRL	KVETYKEQPL	RFYIQMCTEL	TYGPVFMCL
Derived from	Score[a]	14.4	19.8	17.3	14.4		
		IFN-γ production (pg/ml)[b]					
Healthy donors							
#1		154	352	10	394	306	0
#2		156	132	8	17	0	207
#3		497	0	7	20	17	0
#4		0	0	37	2	59	14
#5		184	0	166	38	0	27
#6		1354	0	0	357	124	168
#7		166	38	0	0	1017	0
#8		0	194	0	1017	0	0
#9		0	5624	5	61	123	228
#10		0	168	1354	0	0	3
Total		6/10	5/10	2/10	3/10	4/10	3/10
Cancer patients							
#1		180	154	145	0	0	15
#2		122	138	15	9	5	0
#3		699	8	17	38	0	21
#4		31	105	24	19	0	159
#5		799	28	16	10	130	20
#6		500	4	1	14	198	15
#7		317	0	0	0	ND	ND
#8		4	1060	411	23	115	189
#9		17	101	1	0	709	3
#10		180	198	196	118	40	27
Total		7/10	6/10	3/10	1/10	4/9	2/9

[a]The score represents the estimated half-time of dissociation of the PTHrP peptides binding to HLA-A24 molecules. [b]The PBMCs of HLA-A24$^+$ healthy donors and prostate cancer patients were stimulated *in vitro* with the indicated PTHrP peptide, as described in Material and Methods. On the 15th day, the cultured PBMCs were tested for their reactivity to C1R-A24 cells, which were prepulsed with the corresponding peptide or the HIV peptide. The values represent the mean of two wells, and the background IFN-γ production in response to the HIV peptide was subtracted. Significant values ($P<0.05$ by two-tailed Student's *t*-test) are underlined. ND = not done.

Cytotoxicity assay

After *in vitro* stimulation with the PTHrP peptides, the peptide-stimulated PBMCs were additionally cultured with 100 U ml^{-1} IL-2 for approximately 10 days, in order to obtain a sufficient number of cells to carry out a cytotoxicity assay. These cells were then tested for cytotoxicity against both LNCaP and LNCaP-A24 by a 6-h ^{51}Cr-release assay. A total of 2000 ^{51}Cr-labelled cells per well were cultured with effector cells in 96-round-well plates at the indicated effector/target ratios. In some experiments, either anti-HLA class I (W6/32: mouse IgG2a), anti-HLA-DR (L243: mouse IgG2a), anti-CD4 (NU-TH/I: mouse IgG1), anti-CD8 (NU-TS/C: mouse IgG2a), or anti-CD14 (H14: mouse IgG2a) mAb was added to the wells at a dose of 20 μg ml^{-1} at the initiation of the assay.

Cold inhibition assay

The specificity of the PTHrP peptide-stimulated CTLs was confirmed by a cold inhibition assay. In brief, ^{51}Cr-labelled target cells (2×10^3 cells per well) were cultured with the CTLs (4×10^4 cells per well) in 96-round-well plates with 2×10^4 cold target cells. C1R-A24 cells, which were prepulsed with either the HIV peptide or a corresponding PTHrP peptide, were used as cold targets.

Detection of peptide-specific IgG

The peptide-specific IgG levels in the plasma were measured by ELISA, as previously reported (Nakatsura *et al*, 2002; Ohkouchi *et al*, 2002). In brief, peptide (20 μg per well)-immobilised plates were blocked with Block Ace (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween-20-PBS, after which 100 μl per well of plasma sample diluted with 0.05% Tween-20-Block Ace was added to the plate. After a 2-h incubation at 37°C, the plates were washed and further incubated for 2-h with a 1 : 1000-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 IgG-conjugated horseradish peroxidase (EnVision, DAKO) was added to each well, and the plates were then incubated at room temperature for 40 min. After the plates were washed once, 100 μl per well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of 1 M phosphoric acid. The values are shown as optical density (OD) units ml^{-1}. IgG reactive to a corresponding PTHrP peptide was judged to be positive when the difference of the OD in 1 : 100-diluted plasma exceeded 0.05. To confirm the specificity of IgG to the indicated PTHrP peptide, sample plasma was cultured with plates coated with either the corresponding PTHrP peptide or an irrelevant PTHrP peptide. Thereafter, the levels of PTHrP peptide-specific IgG in the resulting supernatant were determined by ELISA.

Statistics

The statistical significance of the data was determined using a two-tailed Student's *t*-test. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Induction of PTHrP peptide-specific CTLs from HLA-A24$^+$ healthy donors and prostate cancer patients

First, four PTHrP-derived peptides were prepared based on their binding affinity to HLA-A24 molecules (Parker *et al*, 1994; Rammensee *et al*, 1995) (Table 1). Although the PTHrP$_{1-36}$ peptide is a propeptide (Suva *et al*, 1987; Juppner *et al*, 1991), the PTHrP$_{25-34}$ peptide was included. With regard to the difference in amino acids, three amino acids were found to differ between the PTHrP$_{36-44}$ peptide and PTH, and all of the amino acids were found to differ between the other three PTHrP peptides and PTH. Next, to investigate the immunogenicity of these four PTHrP peptides, the PBMCs of 10 HLA-A24$^+$ healthy donors and 10 HLA-A24$^+$ prostate cancer patients were stimulated with each of four PTHrP peptides, and were then examined for their IFN-γ production in response to C1R-A24 cells, which were prepulsed with either a corresponding PTHrP peptide or the HIV peptide (Table 1). Flu- and BEV-derived peptides were used as controls. The assay was carried out in quadruplicate. The cultured cells in one well were separated into four wells. Two wells were used for the PTHrP peptide-pulsed C1R-A24 cells, and the other two wells for the HIV peptide-pulsed C1R-A24 cells. The background IFN-γ production in response to the HIV peptide was subtracted, and the results that showed the best response are shown in Table 1. The successful induction of peptide-specific CTLs was judged to be positive when significant values ($P<0.05$ by two tailed Student's *t*-test) were observed. The results showed that the PTHrP$_{36-44}$, PTHrP$_{102-111}$, PTHrP$_{25-34}$, and PTHrP$_{110-119}$ peptides induced peptide-specific CTLs in six, five, two, and three of 10 HLA-A24$^+$ healthy donors, respectively. These PTHrP peptides also induced peptide-specific CTLs in seven, six, three, and one of 10 HLA-A24$^+$ prostate cancer patients, respectively. The net IFN-γ production of the cases with 10 HLA-A24$^+$ prostate cancer patients in response to the corresponding PTHrP peptide or the HIV peptide are shown in Figure 1. In total, these findings indicate that both the PTHrP$_{36-44}$ and PTHrP$_{102-111}$ peptides are promising candidates to generate peptide-specific CTLs from HLA-A24$^+$ prostate cancer patients.

Induction of prostate cancer-reactive CTLs using PTHrP$_{36-44}$ and PTHrP$_{102-111}$ peptides

In order to investigate the HLA-A24-restricted and prostate cancer-reactive cytotoxicity of peptide-stimulated PBMCs, we prepared an HLA-A24-expressing LNCaP cell line, which we designated LNCaP-A24 (Figure 2). LNCaP has previously been reported to produce PTHrP (Francini *et al*, 2002). A parental LNCaP cell line was negative for the cell surface expression of HLA-A24 molecules, whereas the LNCaP-A24 cell line expressed HLA-A24 molecules on their cell surface. It was then determined whether PBMCs stimulated by either the PTHrP$_{36-44}$ or PTHrP$_{102-111}$ peptide could induce prostate cancer-reactive CTLs from HLA-A24$^+$ healthy donors and prostate cancer patients. PBMCs from HLA-A24$^+$ healthy donors and cancer patients were repeatedly stimulated with the indicated PTHrP peptide, based on the culture protocol described in Materials and Methods. After confirming that these peptide-stimulated cells could produce IFN-γ in response to PTHrP peptide-pulsed C1R-A24 cells, the peptide-stimulated PBMCs were examined for their cytotoxicity against three targets. It was found that the PTHrP peptide-stimulated PBMCs from HD#2, Pt#1, and Pt#2 produced higher levels of IFN-γ in response to the corresponding PTHrP peptide-pulsed C1R-A24 cells than to the HIV peptide-pulsed C1R-A24 cells (Figure 3A). These peptide-stimulated PBMCs also showed higher levels of cytotoxicity against the LNCaP-A24 cell line than against the LNCaP line and HLA-A24$^+$ PHA-induced T cell blasts (Figure 3B). In addition, their cytotoxicity against LNCaP-A24 was significantly inhibited by the addition of anti-HLA-class I and anti-CD8 mAbs, but not by the addition of other anti-HLA-class II, anti-CD4, or anti-CD14 mAbs (Figure 4A). Furthermore, their cytotoxicity against the LNCaP-A24 cell line was significantly suppressed by the addition of the corresponding PTHrP peptide-pulsed C1R-A24 cells, as a cold target, but this suppression was not observed with the addition of HIV peptide-pulsed C1R-A24 cells (Figure 4B). In