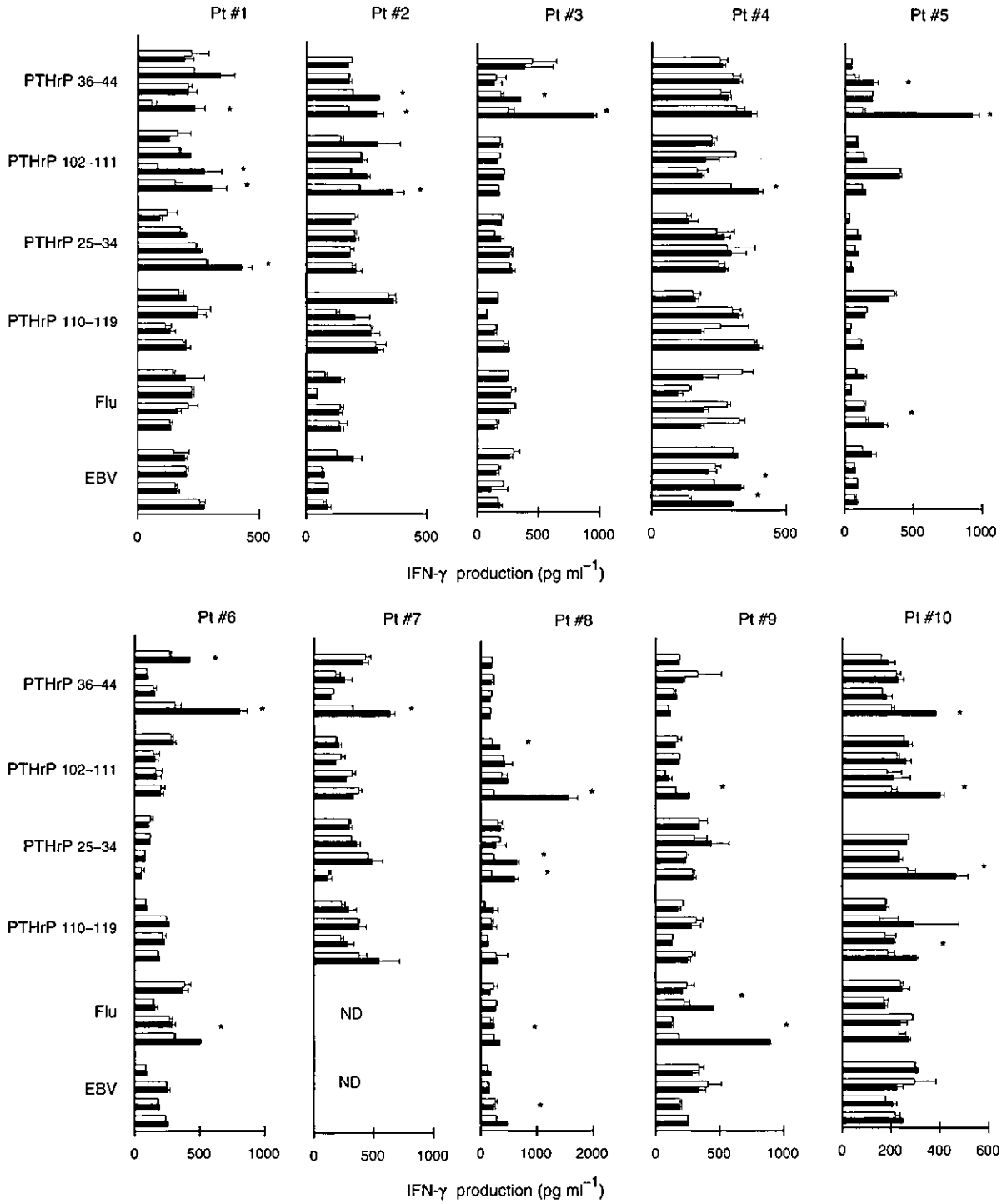

Figure 1 Induction of PTHrP peptide-specific CTLs from the PBMCs of HLA-A24$^+$ prostate cancer patients. PBMCs from 10 HLA-A24$^+$ prostate cancer patients were stimulated *in vitro* with the PTHrP peptides indicated, as described in Materials and Methods. On the 15th day, the peptide-stimulated cells were cultured with C1R-A24 cells, which were prepulsed with an HIV peptide (open bar) and the indicated PTHrP peptide (closed bar) for 18-h. The levels of IFN-γ in the supernatants were then determined by ELISA. *$P<0.05$ was considered statistically significant.

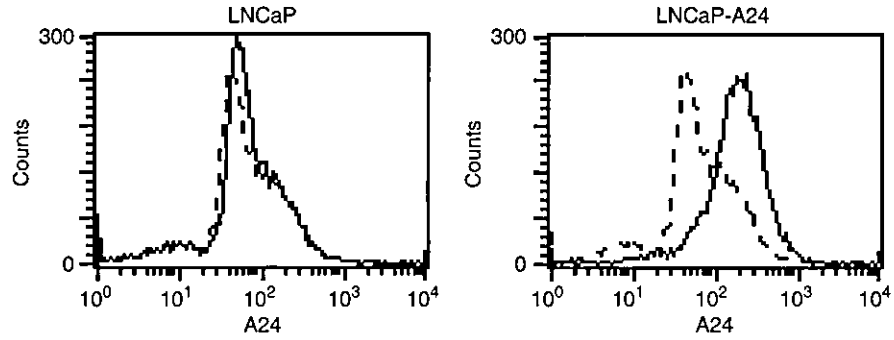

Figure 2 An HLA-A24-expressing LNCaP cell line. Flow cytometric analysis was performed on the LNCaP and LNCaP-A24 cells. These cells were stained with anti-HLA-A24 mAb, followed by FITC-conjugated anti-mouse IgG mAb. The dotted lines represent staining without the first mAb.

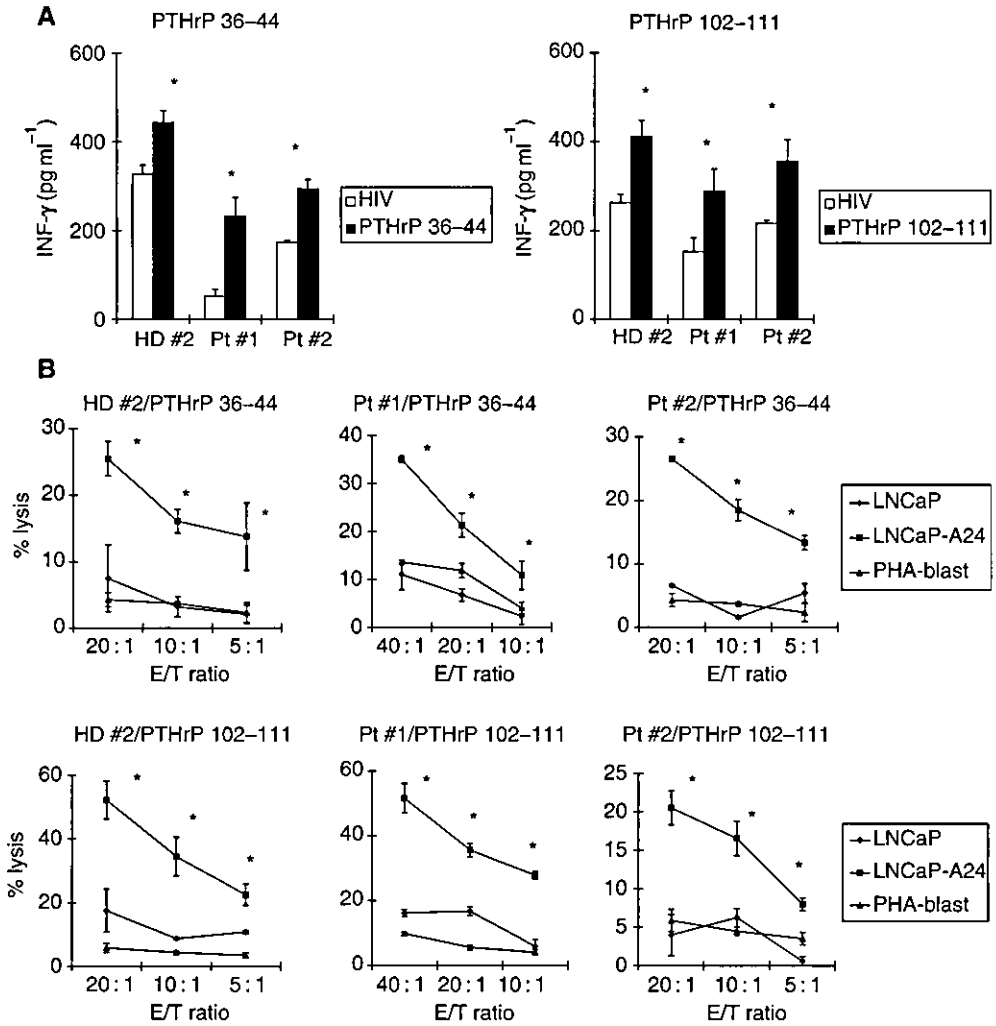

Figure 3 Induction of HLA-A24-restricted and prostate cancer-reactive CTLs from the PBMCs of healthy donors and cancer patients. (**A**) PBMCs from one HLA-A24$^+$ healthy donor (HD #2) and from two HLA-A24$^+$ prostate cancer patients (Pt #1 and Pt #2) were stimulated *in vitro* with the indicated PTHrP peptides, as described in Materials and Methods. On the 15th day, half of the cultured cells were harvested, pooled from four wells, and cultured with C1R-A24 cells, which were prepulsed with an HIV peptide (open symbol) and the indicated PTHrP peptide (closed symbol) for 18-h. The levels of IFN-γ in the supernatants were then determined by ELISA. (**B**) Thereafter, these cells were examined for their cytotoxicity against the LNCaP cells (HLA-A24$^-$), LNCaP-A24 cells (HLA-A24$^+$), and PHA-blastoid T cells (HLA-A24$^+$). A 6-h ^{51}Cr-release assay was performed. Values represent the mean of triplicate assays. *$P<0.05$ was considered statistically significant.

addition, we observed that these PTHrP peptide-stimulated PBMCs from cancer patients showed cytotoxicity against another prostate cancer cells PC-93-A24, stably expressed the HLA-A24 molecules and produced PTHrP (data not shown). These results indicate that both the $PTHrP_{36-44}$ and $PTHrP_{102-111}$ peptides have the potential to induce prostate cancer-reactive CTLs from HLA-A24$^+$ prostate

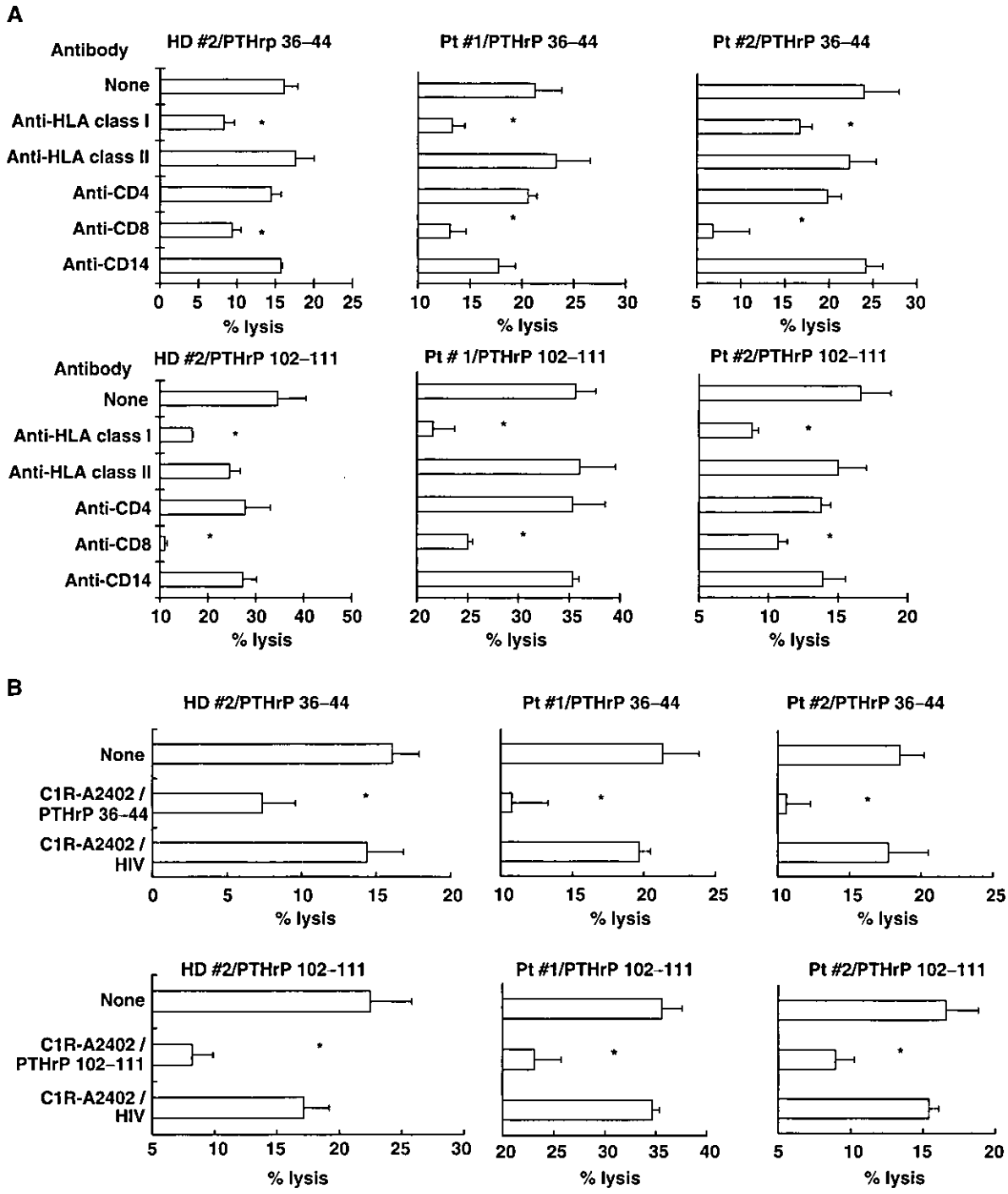

Figure 4 $CD8^+$ T-cell-dependent and PTHrP peptide-specific cytotoxicity against LNCaP-A24 cells. (**A**) The PTHrP peptide-stimulated PBMCs, described in Figure 2, were examined for their cytotoxicity against the LNCaP-A24 cell line, with or without anti-HLA class I, anti-HLA class II, anti-CD4, anti-CD8, or anti-CD14 mAb at a dose of 20 $\mu g\,ml^{-1}$. The values represent the mean of triplicate assays. *$P<0.05$ was considered statistically significant. (**B**) The cytotoxicity against the LNCaP-A24 cell line (2×10^3 cells per well) was also examined in the presence of unlabelled C1R-A24 cells (2×10^4 cells per well), which were prepulsed with the HIV peptide or a corresponding PTHrP peptide. The values represent the mean of triplicate assays. *$P<0.05$ was considered statistically significant.

cancer patients, and that their cytotoxicity against prostate cancer was dependent on PTHrP peptide-specific $CD8^+$ T cells.

Detection of IgG reactive to the PTHrP peptides

We previously reported that IgGs reactive to CTL epitope peptides were detected in healthy donors and cancer patients (Nakatsura *et al*, 2002; Ohkouchi *et al*, 2002). IgGs reactive to prostate-related antigens were also detected in healthy donors and prostate cancer patients (Harada *et al*, 2003a; Kobayashi *et al*, 2003; Matsueda *et al*, 2004) Therefore, we attempted to determine whether IgG reactive to four PTHrP-derived peptides could be detected in the plasma of cancer patients and healthy donors. The result was that IgG reactive to either the $PTHrP_{102-111}$ or the $PTHrP_{109-119}$ peptide

Table 2 IgG reactive to the PTHrP peptides in plasma of HLA-A24$^+$ healthy donors and prostate cancer patients

	Healthy donors											Prostate cancer patients										
Peptides	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**Total**	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**Total**
$PTHrP_{36-44}$	−	−	−	−	−	−	+	+	+	−	3/10	−	−	−	−	+	−	−	−	−	−	1/10
$PTHrP_{102-111}$	+	−	+	+	+	−	+	+	+	+	8/10	+	+	−	−	+	+	+	+	+	−	7/10
$PTHrP_{25-34}$	−	−	−	−	−	−	−	−	−	−	0/10	−	−	−	−	−	−	−	−	−	−	0/10
$PTHrP_{110-119}$	+	+	+	−	+	−	+	+	+	+	8/10	+	+	−	−	−	+	+	+	+	+	7/10

IgG reactive to the corresponding peptide was judged to be positive when the difference in the OD in 1 : 100-diluted plasma exceeded 0.05. The cutoff level (OD: 0.05) was determined based on the levels of anti-HIV peptide IgG in HIV-negative healthy donors.

was detected in eight of 10 healthy donors and in seven of 10 prostate cancers (Table 2). Representative results are in Figure 5A. However, IgG reactive to the $PTHrP_{36-44}$ peptide was detected in three of 10 healthy donors and one of 10 prostate cancer patients, respectively. No IgG reactive to $PTHrP_{25-34}$ was detected in any of the healthy donors or cancer patients. The levels of PTHrP peptide-specific IgG were significantly diminished by culturing the plasma in the corresponding PTHrP peptide-coated wells (Figure 5B). This peptide-specific absorption demonstrated the validity of the present assay system.

DISCUSSION

Prostate cancer appears to be a good target for the development of specific immunotherapies (Harada *et al*, 2003b). In recent years, our group has attempted to identify epitope peptides derived from prostate-related antigens that would be able to generate prostate cancer-reactive CTLs from prostate cancer patients (Inoue *et al*, 2001; Kobayashi *et al*, 2003; Harada *et al*, 2003a; Matsueda *et al*, 2004). However, one major obstacle encountered when treating prostate cancer patients is the treatment of bone metastases, as prostate cancer frequently metastasises to the bone tissue. Therefore, we undertook the present study to identify epitope peptides that could potentially be suitable for specific the immunotherapy of HLA-A24$^+$ prostate cancer patients with metastases.

PTHrP is known to be a key agent in the development of bone metastasis in cases of prostate cancer, and prostate cancer cells has been reported to produce PTHrP (Francini *et al*, 2002). These lines of evidence indicate that PTHrP could be a good target for the development of specific immunotherapies against metastatic prostate cancer. Indeed, $PTHrP_{59-68}$ and $PTHrP_{165-173}$ peptides have been reported to be candidates for such specific immunotherapy of HLA-A2$^+$ prostate cancer patients (Guise, 1997; Francini *et al*, 2002). In this study, we identified new PTHrP peptides that have the potential to generate prostate cancer-specific CTLs in HLA-A24$^+$ prostate cancer patients, in order to extend the possibility of PTHrP peptide-based anticancer vaccine. We revealed that both the $PTHrP_{36-44}$ and the $PTHrP_{102-111}$ peptides have the potential to induce prostate cancer-reactive CTLs in HLA-A24$^+$ prostate cancer patients. PBMCs from HLA-A24$^+$ prostate cancer patients showed peptide-specific IFN-γ production in six or seven of 10 patients when stimulated with the $PTHrP_{102-110}$ and $PTHrP_{36-44}$ peptide, respectively. More importantly, PBMCs that were stimulated with these PTHrP peptides showed cytotoxicity against prostate cancer cells in an HLA-A24-restricted manner. These results indicate that these two PTHrP peptides are immunogenic, and therefore potentially useful for the specific immunotherapy of HLA-A24$^+$ prostate cancer patients with metastases.

The $PTHrP_{36-44}$ and the $PTHrP_{102-110}$ peptides also induced peptide-specific and tumour-reactive CTLs from the PBMCs of HLA-A24$^+$ healthy donors. This result is consistent with that of a previous report demonstrating the induction of PTHrP peptide-specific CTLs from the PBMCs of HLA-A2$^+$ healthy donors (Francini *et al*, 2002). As the $PTHrP_{36-44}$ peptide shares three amino acids with PTH, and because there is no homology between the $PTHrP_{102-111}$ peptide and PTH, crossreactivity between the PTHrP peptides and PTH could be excluded. Low levels of PTHrP have been sporadically detected in keratinocytes, uterus, and mammary glands during lactation (Tian *et al*, 1993). Recent advances in tumour immunology have revealed that self-antigens on human cancer cells are the most prevalent antigens recognized by the immune system (Rosenberg, 1999; Renkvist *et al*, 2001). CTL precursors reactive to nonmutated self-antigens may circulate in the peripheral blood of both certain healthy donors and cancer patients.

Here, we investigated whether or not IgG against PTHrP peptides would be detectable in plasma from HLA-A24$^+$ healthy donors and prostate cancer patients, because the antibodies against CTL epitope peptides had already been observed in certain cancer patients and healthy donors (Nakatsura *et al*, 2002; Ohkouchi *et al*, 2002). We also previously reported that IgG reactive to peptides derived from prostate-related antigens was frequently detectable in healthy donors and prostate cancer patients (Harada *et al*, 2003a; Kobayashi *et al*, 2003; Matsueda *et al*, 2004). In this study, IgG reactive to either the $PTHrP_{102-111}$ peptide or $PTHrP_{110-119}$ peptide was frequently detected in healthy donors as well as in prostate cancer patients. This means that the $PTHrP_{102-111}$ peptide was recognized by both the cellular and humoral immune systems. Although we do not yet have a clear understanding of the roles played by peptide-specific IgG in antitumour immune responses, our clinical trials revealed that a peptide vaccination frequently resulted in the induction of IgG reactive to the CTL epitope peptides which were administered (Noguchi *et al*, 2003; Tanaka *et al*, 2003). In addition, the induction of IgG reactive to the vaccinated peptides was positively correlated with longer survival of advanced lung cancer patients (Mine *et al*, 2003). As regards the use of a peptide vaccination in cases of gastric cancer, prolonged survival has been observed in patients showing not only cellular, but also humoral immune responses to vaccinated peptides (Sato *et al*, 2003). In addition, the induction of IgG reactive to the administered peptides was correlated with a clinical response among patients with recurrent gynecologic cancer (Tsuda *et al*, 2004). Furthermore, we recently analysed 113 vaccinated patients with various types of cancers, and revealed that the augmentation of peptide-specific IgG after peptide vaccination could be a laboratory marker for the prediction of prolonged survival in vaccinated cancer patients compared to the induction of peptide-specific CTLs or the delayed-type hypersensitivity test (Mine *et al*, 2004). Moreover, we recently observed that peptide vaccination with a 9-mer CTL epitope peptide could induce peptide-specific and HLA-DR-restricted CD4$^+$ T cells *in vivo* (Harada *et al*, 2004). As these findings provide circumstantial evidence, further clinical study is needed to

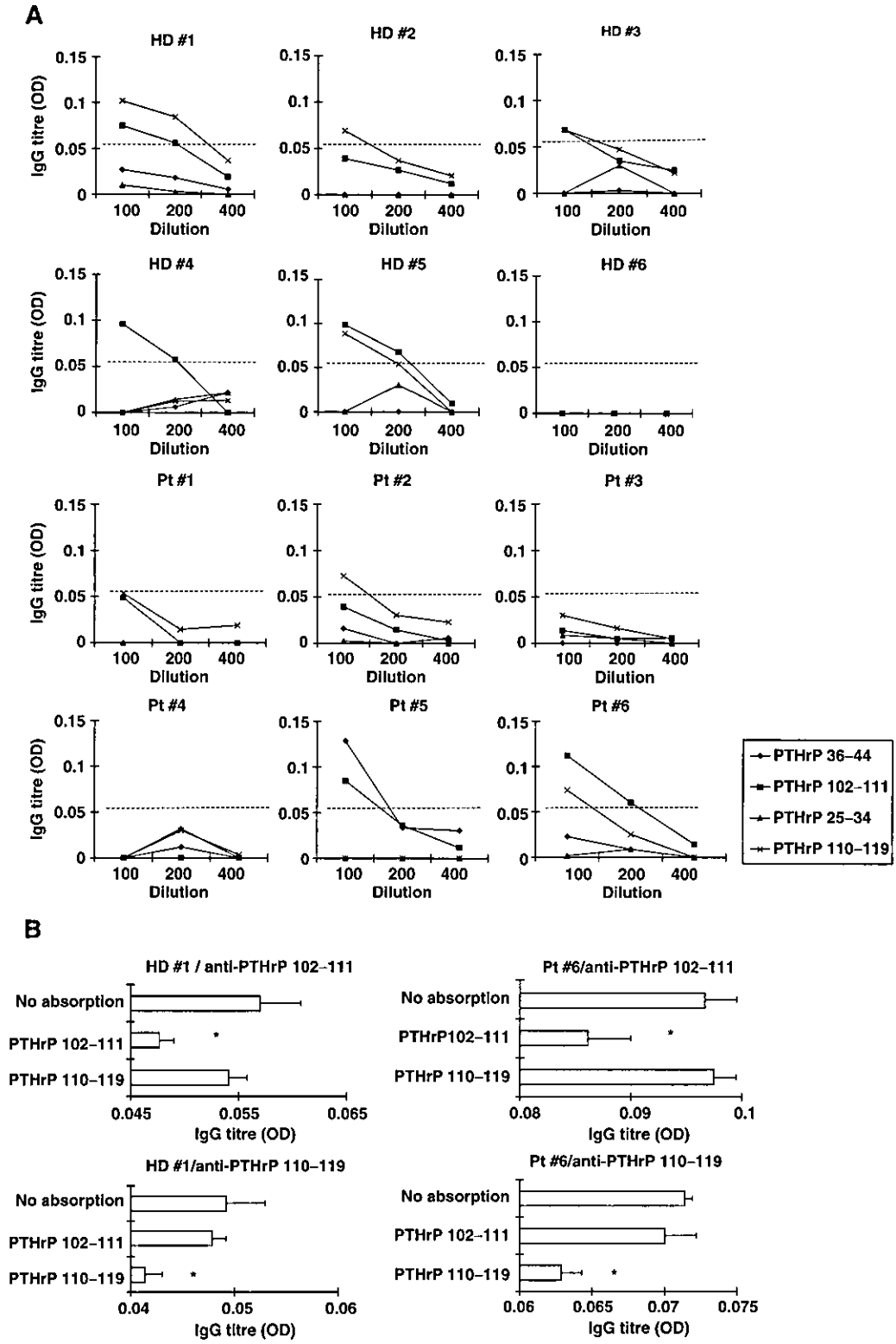

Figure 5 IgG reactive to the PTHrP peptides in plasma from healthy donors and prostate cancer patients. (**A**) Representative results from six healthy donors and six prostate cancer patients are shown. These values are shown as optical density (OD), and the responses to the HIV peptide were subtracted. 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. The cutoff level (OD: 0.05) was determined based on the levels of anti-HIV peptide IgG in HIV-negative healthy donors. (**B**) To confirm the specificity of IgG to the indicated PTHrP peptides, 100 μl of sample plasma from either HD #1 and Pt #6 was cultured in a plate precoated with either a corresponding PTHrP peptide or an irrelevant PTHrP peptide. Thereafter, the levels of IgG reactive to the $PTHrP_{102-111}$ peptide or the $PTHrP_{110-119}$ peptide in the resultant samples were determined by ELISA.

elucidate the role and meaning of peptide-specific IgG in anti-cancer immunotherapy.

In conclusion, we identified new two PTHrP-derived peptides that are immunogenic in HLA-A24$^+$ prostate cancer patients. The frequencies of the HLA-A24 allele are relatively high throughout the world (Imanishi *et al*, 1992). The information provided here might increase the possibility of treating HLA-A24$^+$ prostate cancer patients with metastases using peptide-based immunotherapy.

ACKNOWLEDGEMENTS

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Immediate Early Response Gene X-1, a Stress-Inducible Antiapoptotic Gene, Encodes Cytotoxic T-Lymphocyte (CTL) Epitopes Capable of Inducing Human Leukocyte Antigen-A33-Restricted and Tumor-Reactive CTLs in Gastric Cancer Patients

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ABSTRACT

Peptide-based vaccine therapy, which is designed to elicit T-cell immunity against tumors, is an attractive approach for the treatment of cancer patients. To provide a scientific basis for peptide therapy, an increasing number of CTL-directed peptides have been identified, and some of them have been tried as antigen-specific immunotherapy in the past decade. Only a few studies, however, have been performed on such peptides restricted with alleles other than HLA-A2 and -A24. In the present study, we show that immediate early response gene X-1 (IEX-1), a stress-inducible protein associated with the regulation of cell proliferation and apoptosis, produces antigenic epitopes recognized by 850B-CTLs, HLA-A33-restricted CTLs newly established from T cells infiltrating into gastric adenocarcinoma. The *IEX-1* gene was highly expressed in most cell lines and tissues from various types of cancer at both the mRNA and protein levels. However, it was not expressed at the protein level in any normal epithelium or connective tissues tested. Three IEX-1-derived peptides at positions 47–56, 61–69, and 65–73, which were recognized by the 850B-CTLs, could induce $CD8^+$ peptide-specific CTL reaction to tumor cells from HLA-$A33^+$ gastric cancer patients and other epithelial cancer patients, but not from healthy donors, in an HLA class I-restricted manner. Because increased expression of *IEX-1* is suggested to be involved in the resistance to apoptosis and in the proliferation of cancer cells, these antigenic peptides could be potent candidates for peptide-based specific immunotherapy against HLA-$A33^+$ gastric cancer and other epithelial cancers.

INTRODUCTION

There is growing evidence that human tumors express antigenic peptides recognized by CTLs, and some of these peptides have been used as peptide vaccines for cancer patients with HLA-A2 or -A24 alleles (1–7). In contrast to the many reports on epitope peptides recognized by HLA-A2 or -A24 CTLs (8–11), information on the antigens and peptides recognized by HLA-A33-restricted CTLs is very limited (12, 13). This lack of information is hampering the development of a peptide-based specific immunotherapy for HLA-$A33^+$ cancer patients, regardless of the relatively wide expression of the HLA-A33 allele in various ethnic groups around the world (14, 15). Gastric cancer (GC) is one of the most commonly occurring malignancies in the world (16). The prognosis of this disease is generally good if it is detected at an early stage, but the prognosis for the disease when discovered at an advanced stage, particularly for scirrhous-type cancer, is extremely poor despite recent significant progress in conventional therapeutic modalities. The development of novel therapeutic modalities, such as peptide-based specific immunotherapy, is therefore needed for the treatment of patients at advanced stages of disease. Indeed, we recently reported that peptide vaccination prolonged the overall survival of HLA-A24 or -A2 patients with scirrhous-type GC (4). To identify CTL epitope peptides, we have established a new HLA-A33-restricted CTL line from the tumor-infiltrating lymphocytes (TILs) of a patient with scirrhous-type GC and identified a new gene, *immediate early response gene X-1 (IEX-1*; Ref. 17), which codes for tumor antigens. We have demonstrated that the three IEX-1-derived peptides are capable of inducing HLA-A33-restricted CTL activity reactive to tumor cells in the peripheral blood mononuclear cells (PBMCs) of patients with epithelial cancer. The identified antigenic peptides may be clinically useful as appropriate target molecules in specific immunotherapy for HLA-$A33^+$ cancer patients.

MATERIALS AND METHODS

Generation of the 850B-CTL Line. The HLA-A33-restricted and tumor-specific CTL line (850B-CTL) was newly established from the TILs of a patient with scirrhous-type GC (HLA-A*2402/A*3303, B7/B44, Cw7/Cw14) by the method reported previously (11). The established CTL line was tested for its responses to various cancer and normal cells by use of a 6-h ^{51}Cr-release assay and by measurement of IFN-γ with an ELISA, as reported previously (11). The surface phenotype of the CTL line was examined by an immunofluorescence assay with FITC-conjugated anti-CD3, -CD4, or -CD8 monoclonal antibodies (mAbs). For the inhibition of CTL activity, 100 μg/ml each of anti-HLA class I (W6/32, IgG2a); anti-CD8 (Nu-Ts/c, IgG2a); anti-HLA-A24 (0041HA, IgG2a); anti-CD4 (Nu-Th/i, IgG1); anti-HLA class I B, C (B1–23, IgG2a); anti-HLA class II (H-DR1, IgG2a); and anti-CD14 (JML-H14, IgG1) mAbs were used as reported previously (11). The cancer cell lines used for this experiment were as follows: gastric adenocarcinoma (MKN-28, MKN-45, SSTW-9, KATO-III, KWS, and HGC-27), lung carcinoma (LC-1 and QG-56), head and neck carcinoma (KUMA-1), colon adenocarcinoma (SW620 and COLO 201), pancreatic adenocarcinoma (Panc-1), and human chronic myelogenous leukemia (K562). The HLA class I genotypes of these tumor cells are described elsewhere (11). The expression of HLA class I or HLA-A33 antigens on these cells was measured by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA) after the cells were stained with anti-HLA class I (W6/32) mAb, which recognizes a monomorphic region of HLA class I molecule, or anti-HLA-A33 mAb (IgM; One Lamda, Canoga Park, CA), which recognizes a polymorphic region of HLA-A33 molecule as reported previously (13).

Identification of the *IEX-1* Gene. An expression gene-cloning method was used to identify the gene coding for the tumor antigen recognized by the 850B-CTL line, as reported previously (11). cDNA from LC-1 was inserted into the expression vector pSV-SPORT-6 (Invitrogen, San Diego, CA). *HLA-A*3303* or *HLA-A*2601* cDNA was obtained by reverse transcription-PCR with RNA from KUMA-1 or KE-4 cells, respectively, and was cloned into the eukaryotic expression vector pCR3.1 (Invitrogen). DNA sequencing was performed by a dideoxynucleotide sequencing method using a DNA sequence kit and ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster, CA).

Northern Blot Analysis and Immunohistochemistry. The expression of *IEX-1* mRNA on various tumor or normal tissues (Multiple Tissue Northern

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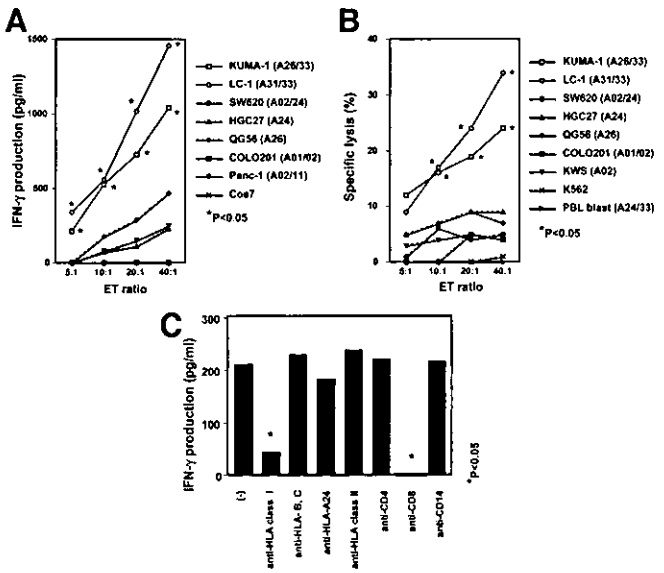

Fig. 1. Characterization of the HLA-A33-restricted 850B-CTL cell line. *A*, the 850B-CTL cell line was tested for its ability to produce IFN-γ in response to a panel of target cells at various E:T ratios. *Values* represent the means of triplicate determinations. *B*, the cytotoxic activity of 850B-CTLs against various target cells was tested by a 6-h ^{51}Cr-release assay at different E:T ratios. *Values* represent the means of triplicate determinations. *PBL*, peripheral blood lymphocytes. *C*, IFN-γ production by 850B-CTLs in response to LC-1 tumor cells was tested in the presence of 100 μg/ml each of anti-HLA class I; anti-HLA class I B, C; anti-HLA-A24; anti-HLA class II; anti-CD4; anti-CD8; or anti-CD14 monoclonal antibodies. *, $P < 0.05$.

Blots; Clontech, Tokyo, Japan) was examined by Northern blot analysis with a ^{32}P-labeled *IEX-1* probe as reported previously (9). The expression of IEX-1 protein was evaluated by immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections, with use of a Ventana Medical Systems automated instrument (Tucson, AZ) with anti-IEX-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Determination of Antigenic Peptides. Among possible peptide sequences with motifs for binding to the HLA-A33 molecule (18, 19) in the deduced amino acid sequence of IEX-1, eight different peptides that showed stronger binding activity for HLA-A33 in a computer analysis (Bioinformatics and Molecular Analysis Section, NIH, Bethesda, MD) were used. Peptides with a purity of >95% were obtained from Biologica (Nagoya, Japan). For the peptide-binding assay, RMA-S-A33 cells, RMA-S tap (transporter-associated peptide processing)-deficient mouse lymphoma cells stably transfected with *HLA-A*3303* cDNA, were used. Briefly, the cells were incubated at 26°C for 18 h and were suspended in Opti-MEM containing 3 μg/ml human β_2-microglobulin and 100 μg/ml peptides, followed by incubation at 26°C for 3 h and at 37°C for 3 h. The cells were then incubated with anti-HLA-A33 mAb at 4°C for 30 min, followed by incubation with FITC-conjugated rabbit antimouse IgM antibody (Cappel, Aurora, OH) at 4°C for 30 min. The cells were analyzed by FACScan, and their binding activity was evaluated by the mean fluorescence intensity. Cells pulsed with TRP2–197 peptide were used as a control (12). For the detection of antigenic peptides recognized by the 850B-CTL line, C1R-A33 cells were used as reported previously (13). IFN-γ production in the culture supernatants was measured by an ELISA.

Induction of CTLs by Peptides. After written informed consent was obtained, PBMCs from HLA-A33$^+$ cancer patients (n = 4; 2 GC patients, 1 lung cancer patient, and 1 prostate cancer patient), and 5 HLA-A33$^+$ healthy donors served as subjects for the CTL induction assay. PBMCs (1×10^5 cells/well) were incubated with each peptide in the wells of a 96-well microculture plate (Nunc, Roskilde, Denmark), as reported previously (13). On the 14th day of the culture, the cells were tested for their ability to produce IFN-γ in response to C1R-A33 pulsed with a corresponding peptide or a negative-control peptide (HIV). After an 18-h incubation, the supernatant was collected for the measurement of IFN-γ by ELISA. The PBMCs showing a positive response were further cultured with interleukin-2 alone for 10–14 days for a standard 6-h ^{51}Cr-release assay (13). For the inhibition test, 20 μg/ml each 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.

Statistical Analysis. A two-tailed Student's t test was used for statistical analysis throughout the study.

RESULTS

Establishment of an HLA-A33-Restricted, Tumor-Specific CTL Line. A CTL line (850B-CTL) was established from the TILs of a GC patient and was characterized by testing its reactivity to various cancer and normal cells as determined by both IFN-γ production and 6-h ^{51}Cr-release assay. As shown in Fig. 1*A*, this CTL line produced significant levels of IFN-γ in the presence of the HLA-A33$^+$ epithelial cancer cell lines LC-1 and KUMA-1 and lower levels of IFN-γ in response to QG56 (A26), Panc-1 (A02/11), and HGC27 (A02/24); it did not produce any IFN-γ in response to the other three HLA-A33$^-$ target cell lines tested. The 850B-CTL cell line also showed significant levels of cytotoxicity against LC-1 and KUMA-1 cells but not against any of the five HLA-A33$^-$ target cells, COS-7 cells, the natural killer target cell line K562, or HLA-A33$^+$ phytohemagglutinin-activated normal T cells (phytohemagglutinin-blast cells) from the PBMCs of healthy donors (Fig. 1*B*). The expression levels of HLA-A33 of these tumor cells were not largely different from those of the normal cells (data not shown). The production of IFN-γ by the 850B-CTL cell line in response to HLA-A33$^+$ LC-1 cells was significantly inhibited by 100 μg/ml anti-HLA class I or anti-CD8 mAb but not by anti-HLA-BC, anti-HLA class II, anti-HLA-A24, anti-CD4, or an isotype-matched irrelevant anti-CD14 mAb (Fig. 1*C*). The phenotype of the 850B-CTL cell line (>98%) was CD3$^+$CD4$^-$CD8$^+$ (data not shown). These results indicate that the 850B-CD8$^+$ CTL line largely consisted of T cells with HLA-A33-restricted, tumor-reactive

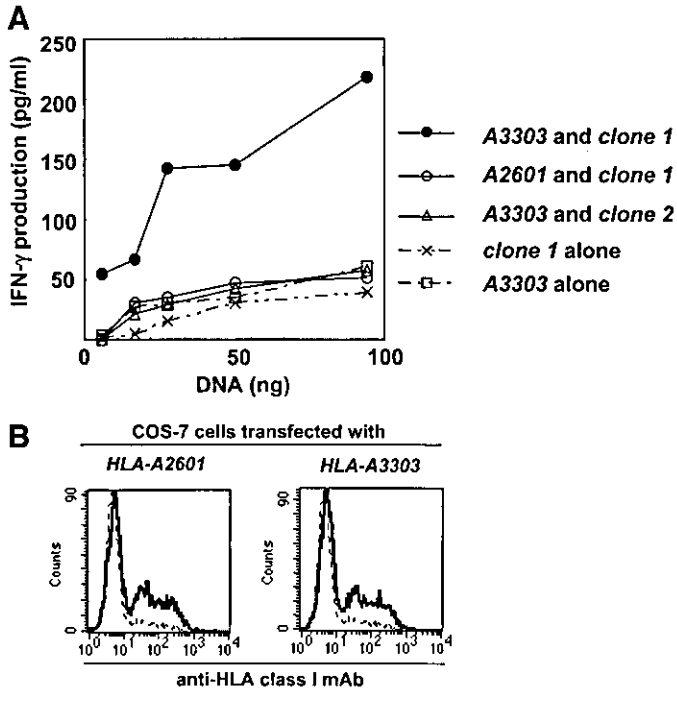

Fig. 2. HLA-A33-restricted recognition of an identified tumor antigen gene by the 850B-CTL cell line. *A*, COS-7 cells were transfected with the indicated amounts of *clone 1* or *clone 2* and 100 ng of *HLA-A*3303* or *HLA-A*2601*, and their stimulatory effects on IFN-γ production by 850B-CTLs were tested. *Values* represent the means of triplicate determinations. *B*, COS-7 cells were transfected transiently with *HLA-A*2601* or *-A*3303* cDNA, and their expression levels were analyzed by FACScan with anti-HLA class I monoclonal antibody (*mAb*). *Dotted line* indicates the isotype-matched negative control. The transfection efficiency was ~30% of total cells with this method.

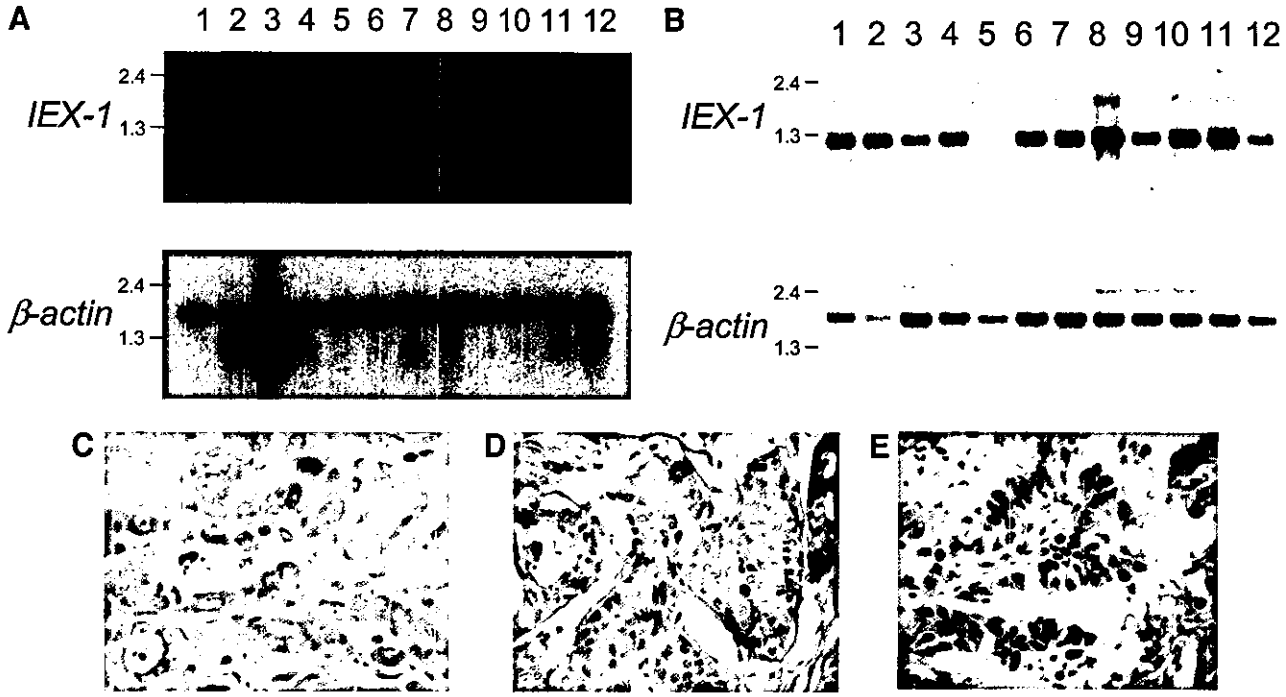

Fig. 3. Expression of immediate early response gene X-1 (*IEX-1*) at the mRNA and protein levels. *A*, expression of *IEX-1* or control *β-actin* mRNA in normal tissues was examined by Northern blot analysis. *Lane 1*, brain; *Lane 2*, heart; *Lane 3*, skeletal muscle; *Lane 4*, colon; *Lane 5*, thymus; *Lane 6*, spleen; *Lane 7*, kidney; *Lane 8*, liver; *Lane 9*, small intestine; *Lane 10*, placenta; *Lane 11*, lung; *Lane 12*, peripheral blood lymphocytes. *B*, expression of *IEX-1* or control *β-actin* mRNA in a panel of cancer cell lines was examined by Northern blot analysis. *Lane 1*, peripheral blood lymphocytes; *Lane 2*, MKN 45; *Lane 3*, MKN 28; *Lane 4*, SSTW; *Lane 5*, HGC 27; *Lane 6*, LC-1; *Lane 7*, QG56; *Lane 8*, KUMA-1; *Lane 9*, Panc-1; *Lane 10*, SW620; *Lane 11*, COLO 201; *Lane 12*, KATO-III. *C–E*, the expression of IEX-1 protein in various tumor tissues was evaluated by immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections, with use of a Ventana Medical Systems automated instrument (Tucson, AZ) with anti-IEX-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Representative photographs of gastric cancer (*C*), breast cancer (*D*), and non-small lung cancer tissues (*E*) are shown.

cytotoxicity, although it contained a few T cells that produced IFN-γ without apparent HLA class I-A restriction or with HLA-A24 restriction.

Identification of a Gene Recognized by 850B-CTLs. A total of 10×10^4 cDNA clones from the cDNA library of LC-1 tumor cells were tested for their ability to stimulate IFN-γ production by the 850B-CTL cell line when cotransfected with *HLA-A*3303* into COS-7 cells. After repeated experiments for several candidate clones, one clone (*clone 1*) was confirmed to encode a tumor antigen recognized by the HLA-A33-restricted 850B-CTLs. As shown in Fig. 2*A*, COS-7 cells transfected with clone 1 and *HLA-A*3303*, but not with *clone 1* and *HLA-A*2601* as a negative control, induced IFN-γ production in 850B-CTLs in a dose-dependent manner. The expression of HLA class I antigens by HLA-A26-transfected COS-7 cells was not significantly different from expression by HLA-A33-transfected COS-7 cells when stained with anti-HLA-class I mAb (Fig. 2*B*). In contrast, COS-7 cells transfected with either *clone 1* or *HLA-A*3303* alone were not recognized by the 850B-CTLs (Fig. 2*A*). The other clones from the LC-1 cDNA library also failed to induce IFN-γ production in 850B-CTLs when cotransfected with *HLA-A*3303* into COS-7 cells. The results of the experiments on *clone 2*, as a negative control, are shown in Fig. 2*A*. The nucleotide sequence of *clone 1* was found by a search of GenBank (GenBank accession no. NM003897) to be completely identical to that of *IEX-1*, which has been reported to be a stress-inducible antiapoptotic gene (17).

Overexpression of *IEX-1* Gene in Tumor Tissues. The expression of *IEX-1* mRNA in normal and cancer cells was investigated by Northern blot analysis. As shown in Fig. 3*A*, a band of ~1.3 kb was clearly detected in all of the normal tissues tested except the brain (Fig. 3*A*, *Lane 1*), with much higher expression in the heart (Fig. 3*A*, *Lane 2*), kidney (Fig. 3*A*, *Lane 7*), lung (Fig. 3*A*, *Lane 11*), and peripheral blood lymphocytes (Fig. 3*A*, *Lane 12*), and lower expression in the thymus (Fig. 3*A*, *Lane 5*), spleen (Fig. 3*A*, *Lane 6*), liver (Fig. 3*A*, *Lane 8*), and small intestine (Fig. 3*A*, *Lane 9*). In addition, this gene was highly expressed in most of the adenocarcinoma and squamous cell carcinoma cell lines tested, except for the HCG27 GC cell line (Fig. 3*B*, *Lane 5*), from various organs, including the stomach (Fig. 3*B*, *Lanes 2–4* and *12*), lung (Fig. 3*B*, *Lanes 6* and *7*), head and neck (Fig. 3*B*, *Lane 8*), pancreas (Fig. 3*B*, *Lane 9*), and colon (Fig. 3*B*, *Lanes 10* and *11*). These results indicate that this gene is overexpressed in the majority of cancer cells and also is expressed in most normal tissues at various levels. To determine the expression of *IEX-1* at the protein level, immunohistochemistry was performed in cancer tissues from various organs. As shown in Fig. 3*C*, the IEX-1 protein was selectively expressed in cytoplasm of all of the GC cells, but not in the surrounding normal epithelium or connective tissues. IEX-1 protein was also selectively expressed in the cytoplasm of the various

Table 1 *Binding activity of immediate early response gene X-1-1-derived peptides to RMA-S-A33 cells*[a]

Peptide	MFI[b]
(−),[c] 26°C	16.4
(−), 37°C	4.2
IEX20–28	14.3
IEX43–51	17.1
IEX47–56	15.2
IEX53–61	14.2
IEX54–63	14.2
IEX61–69	14.4
IEX64–73	14.7
IEX65–73	13.8
TRP2–197	16.0

[a] Binding activity of immediate early response gene X-1-derived peptides to HLA-A33 molecules was evaluated by the mean fluorescence intensity after staining of the RMA-S-A33 cells pulsed with the indicated peptide with anti-HLA-A33 monoclonal antibody.
[b] MFI, mean fluorescence intensity; IEX, immediate early response gene X-1.
[c] (−) indicates no-peptide control.

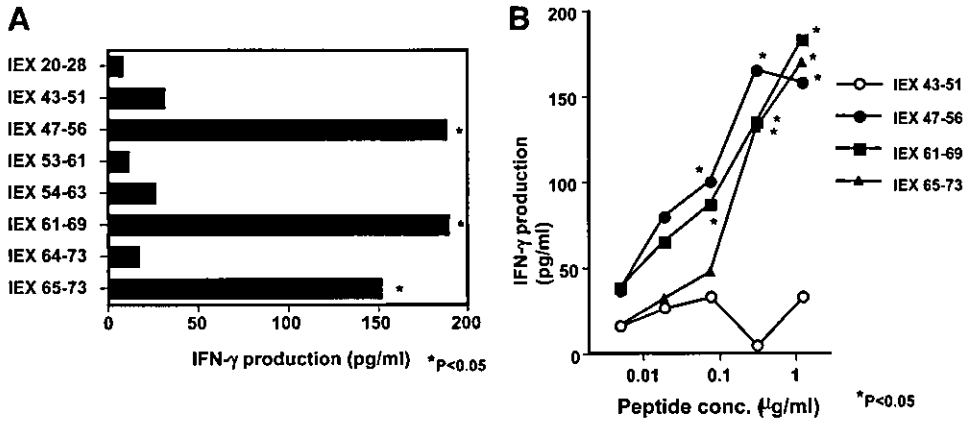

Fig. 4. Identification of CTL epitopes. *A*, determination of the antigenic peptides in immediate early response gene X-1 (IEX-1). The 850B-CTL cells were cultured with the peptide-loaded C1R-A33 for 18 h, and the culture supernatant was harvested to measure IFN-γ by an ELISA. The background IFN-γ production by 850B-CTL in response to peptide-unloaded C1R-A33 cells was subtracted from the values. Values represent the means of triplicate assays. *, $P < 0.05$. *B*, dose-dependent production of IFN-γ by 850B-CTL in response to each of the three IEX-1 peptides. Indicated doses of the IEX-1-derived peptides were loaded on C1R-A33 cells, and the ability of the peptides to stimulate IFN-γ production by the 850B-CTL cells was tested. The background of IFN-γ production by 850B-CTLs in response to peptide-unloaded C1R-A33 cells was subtracted from the values. Values represent the means of triplicate assays. A two-tailed Student's *t* test was used for the statistical analysis. *Conc*, concentration.

types of cancer tissues, including breast (Fig. 3*D*), lung (Fig. 3*E*), and colon (data not shown) cancer tissues. In contrast, it was not expressed in the surrounding normal epithelium or connective tissues of breast, lung, and colon. The only exception among normal cells tested was TILs, which expressed the IEX protein (data not shown).

Identification of IEX-1-Derived Antigenic Peptides Recognized by 850B-CTLs. To identify the IEX-1-derived CTL epitopes, we determined eight possible peptide sequences with motifs for binding to the HLA-A33 molecule in the deduced amino acid sequence of IEX-1 in a computer analysis. Each of the eight synthetic peptides was loaded into HLA-A33-transfected cells (RMA-S-A33) at a concentration of 100 μM, and the binding affinities of these peptides were analyzed. As shown in Table 1, all eight peptides and a reference peptide (TRP2-197) were able to bind to RMA-S-A33 cells, but with slightly different affinities. We next incubated C1R-A33 cells with these eight peptides at a concentration of 1 μM and tested their ability to induce IFN-γ production by the 850B-CTLs. Three of these peptides, IEX47–56, IEX61–69, and IEX65–73, induced significant levels of IFN-γ production (Fig. 4*A*) in a dose-dependent manner (Fig. 4*B*). The optimum concentrations varied for each peptide and were in the range of 0.1–1 μM (Fig. 4*B*); the optimum concentration was not dependent on peptides' binding affinities for the HLA-A33 molecule, determined with RMA-S-A33 cells (Table 1).

Induction of CTLs by IEX-1-Derived Peptides. IEX47–56, IEX61–69, and IEX65–73 peptides were then tested for their ability to induce HLA-A33-restricted, tumor-specific CTLs in the PBMCs of four HLA-A33$^+$ epithelial cancer patients (gastric, lung, or prostate cancer). The PBMCs from these cancer patients, stimulated by each of these three peptides, in most cases tested produced significant amounts of IFN-γ in response to C1R-A33 cells loaded with the corresponding peptides (Fig. 5). In contrast, PBMCs from none of the five healthy donors tested produced significant amounts of IFN-γ in response to C1R-A33 cells loaded with the corresponding peptides even after stimulation with each of these peptides (data not shown).

We next examined CTL activity against tumor cells in the patients' PBMCs stimulated with IEX-1-derived peptides by use of a 6-h ^{51}Cr-release assay. The PBMCs stimulated with each of the three IEX-1-derived peptides, but not those with a negative control peptide (IEX43-51), showed significant levels of cytotoxicity against the HLA-A33$^+$IEX-1$^+$ LC-1 tumor cells, but not against the HLA-A33$^-$ HGC27 or QG56 cells in any of the cases tested (Fig. 6*A*). In contrast, PBMCs stimulated with a negative control peptide (IEX43-51) showed no specific CTL activity (Fig. 6*A*). Collectively, each of the IEX47–56, IEX61–69, and IEX65–73 peptides had the ability to induce HLA-A33-restricted CTL activity toward tumor cells in PBMCs in most of the cases tested, indicating that the three peptides identified, IEX47–56, IEX61–69, and IEX65–73, are antigenic epitopes capable of inducing HLA-A33-restricted, tumor-specific CTLs in PBMCs from patients with epithelial cancer.

The restriction and peptide specificity of the cytotoxicity were confirmed by inhibition and competition assays, respectively. In all of the cases tested, the levels of cytotoxicity of these peptide-stimulated PBMCs were significantly inhibited by anti-HLA class I or anti-CD8 mAb, but not by the other mAbs tested (Fig. 6*B*). The cytotoxicity was also inhibited by the addition of the corresponding peptide-pulsed C1R-A33 cells, but not by addition of the HIV peptide-pulsed cells in all of the cases tested (Fig. 6*C*). These results suggest that the CTL activity against tumor cells was largely mediated by the peptide-reactive CD8$^+$ T cells in an HLA class I-restricted manner.

DISCUSSION

The present study demonstrates that IEX-1 encodes tumor antigenic epitopes recognized by HLA-A33-restricted, tumor-specific CTLs. *IEX-1*, also known as *p22/PRG1* (20), *Dif-2* (21), or the mouse homolog

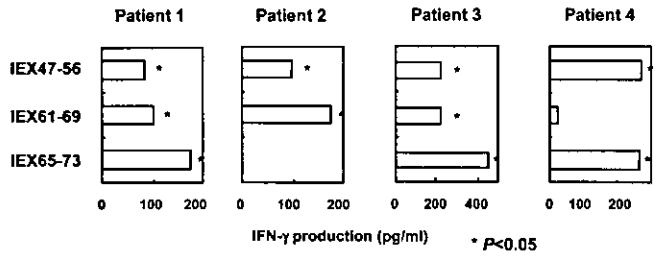

Fig. 5. Cellular response to peptide. Peptide-stimulated peripheral blood mononuclear cells from HLA-A33$^+$ epithelial cancer patients ($n = 4$) and healthy donors ($n = 5$) were tested for their ability to produce IFN-γ in response to C1R-A33 cells pulsed with a corresponding peptide. Background IFN-γ production in response to the HIV peptide (< 50 pg/ml) was subtracted. *, $P < 0.05$, Student's *t* test. The representative results of all four patients are shown. Peptide-specific IFN-γ was not produced by the peripheral blood mononuclear cells from any of the healthy donors tested; thus the results are not shown in the figure. *IEX*, immediate early response gene X-1.

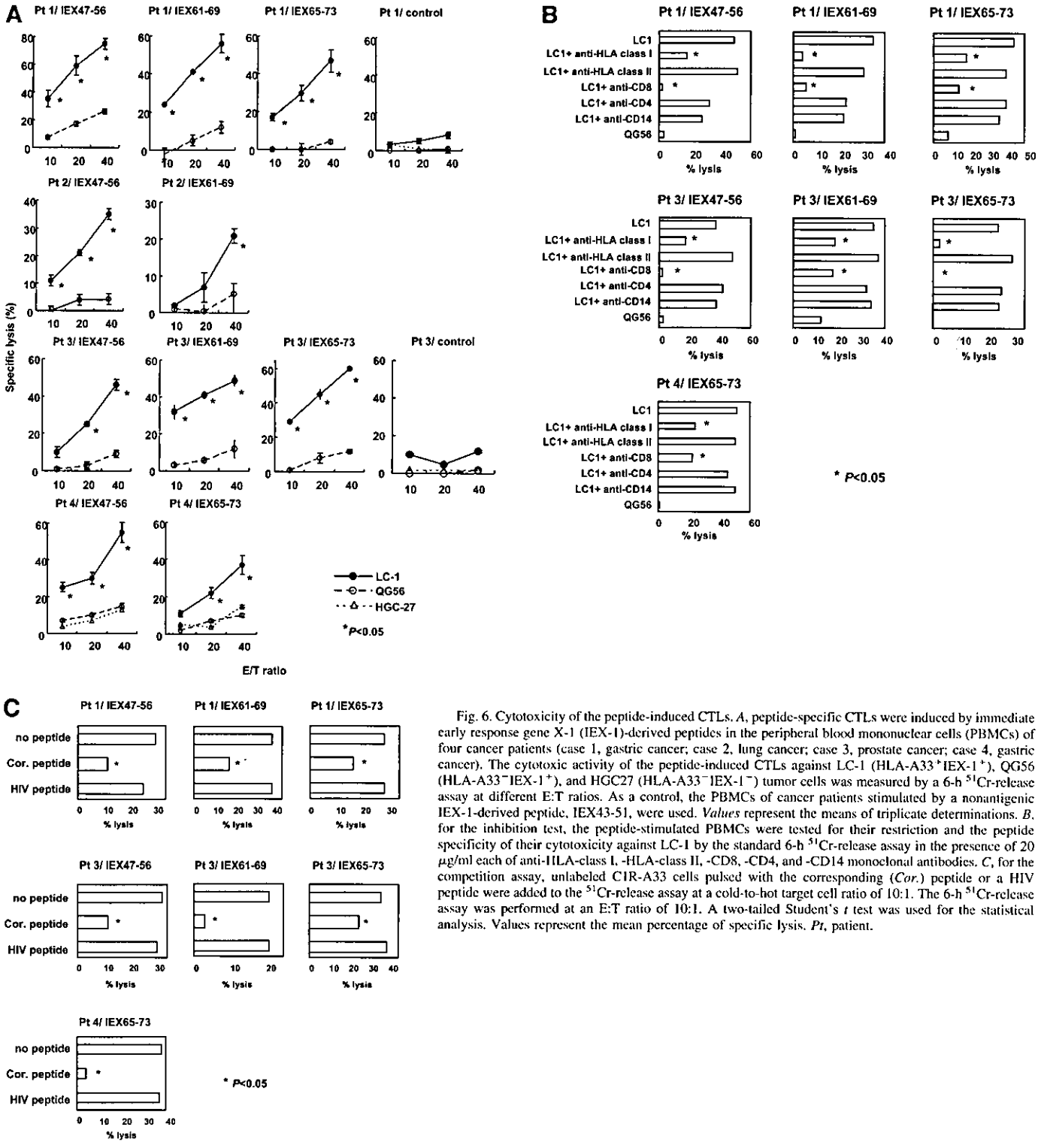

Fig. 6. Cytotoxicity of the peptide-induced CTLs. *A*, peptide-specific CTLs were induced by immediate early response gene X-1 (IEX-1)-derived peptides in the peripheral blood mononuclear cells (PBMCs) of four cancer patients (case 1, gastric cancer; case 2, lung cancer; case 3, prostate cancer; case 4, gastric cancer). The cytotoxic activity of the peptide-induced CTLs against LC-1 (HLA-A33$^+$IEX-1$^+$), QG56 (HLA-A33$^-$IEX-1$^+$), and HGC27 (HLA-A33$^-$IEX-1$^-$) tumor cells was measured by a 6-h ^{51}Cr-release assay at different E:T ratios. As a control, the PBMCs of cancer patients stimulated by a nonantigenic IEX-1-derived peptide, IEX43-51, were used. *Values* represent the means of triplicate determinations. *B*, for the inhibition test, the peptide-stimulated PBMCs were tested for their restriction and the peptide specificity of their cytotoxicity against LC-1 by the standard 6-h ^{51}Cr-release assay in the presence of 20 μg/ml each of anti-HLA-class I, -HLA-class II, -CD8, -CD4, and -CD14 monoclonal antibodies. *C*, for the competition assay, unlabeled C1R-A33 cells pulsed with the corresponding (*Cor.*) peptide or a HIV peptide were added to the ^{51}Cr-release assay at a cold-to-hot target cell ratio of 10:1. The 6-h ^{51}Cr-release assay was performed at an E:T ratio of 10:1. A two-tailed Student's *t* test was used for the statistical analysis. Values represent the mean percentage of specific lysis. *Pt*, patient.

gly96 (22), is a stress-inducible gene and is involved in the regulation of cell cycle progression and apoptosis (17). IEX-1 has been reported to play a key role in the cellular resistance to apoptosis induced by various apoptotic triggers, such as tumor necrosis factor and Fas (23), and to accelerate cell cycle progression in some cell lines (24–26). *IEX-1* transgenic mice show decreased apoptosis in activated T cells, resulting in the accumulation of effector/memory-like T cells and the development of splenomegaly and lymphadenopathy, and are more susceptible to the development of a lupus-like autoimmune disease than their non-transgenic littermates (27). Garcia *et al.* (28) showed that IEX-1 is a new type of substrate for extracellular signal-regulated kinase, one of the mitogen-activated protein kinases, and has a dual role in extracellular signal-regulated kinase signaling by acting both as an extracellular signal-regulated kinase downstream effector mediating survival and as a regulator of extracellular signal-regulated kinase activation. These findings suggest that increased expression of IEX-1 may contribute to malignant transformation in cancer cells through a lack of apoptosis and/or enhanced proliferation.

Expression of IEX-1, a stress-inducible protein, can be rapidly activated by several cellular stresses, including irradiation, growth factors, viral infection, inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β, lipopolysaccharides, and steroid hormones (17). Although *IEX-1* was originally identified as a nuclear factor-κB/rel target gene (23), the *IEX-1* promoter contains several consensus sequences for other transcription factors, such as *p53*, *SP-1*, and *c-Myc* (17). For example, recent studies have suggested that mutation of *p53*, which is common in tumor cells, up-regulates *IEX-1* expression (29). In the present study, we have shown that IEX-1 is highly expressed in most of the cancer cell lines or cancer tissues tested at both the mRNA and protein levels. In contrast, IEX-1 was undetectable at the protein level in any of the normal tissues tested regardless of its expression in normal tissues at the mRNA level. In view of the finding that IEX-1 is strongly expressed in rapidly growing cells (25), IEX-1 may be one of the ideal target molecules in the treatment of patients with cancer. In particular, because irradiation and several chemotherapeutic drugs have been reported to induce IEX-1 expression at relatively high levels (26, 30), a novel therapy, such as a specific immunotherapy, that targets the IEX-1 molecule may be an attractive approach to the treatment of patients with chemotherapy- or radiotherapy-resistant cancers.

Because *IEX-1* is also expressed in normal tissues at the mRNA level, particularly in the heart, kidney, lung, and peripheral blood lymphocytes, these organs may be possible targets for the adverse effects of specific immunotherapy with IEX-1-derived antigenic epitopes. However, the present study showed that neither the 850B-CTL line nor the CTLs induced by the IEX-1-derived peptides lysed phytohemagglutinin-activated normal HLA-A33$^+$ T cells, even in the presence of excess amounts of the corresponding peptide in culture. No severe adverse effects on normal tissues or organs have been observed in our clinical trials with peptide vaccines derived from tumor rejection antigens, some of which are ubiquitously expressed in normal tissues and organs (4–7). Processing of the antigenic peptides in proteasomes or post-translational modification of the peptides in cancer cells may be a little different from these processes in normal cells (8, 31). Further studies, including determination of the molecules involved in the resistance to lysis in normal cells, will be needed to clarify these issues.

The HLA-A33-restricted CTL line used in this study was established from TILs of a GC patient (HLA-A*2402/A*3303) and consisted of bulk-cultured CTLs. Therefore, this CTL line should contain a few CTLs reactive to HLA-A24$^+$ tumor cells. However, the cross-reactivity of this CTL line with HLA-A24 molecules could be functionally negligible because anti-HLA-A24 mAb scarcely inhibited IFN-γ production by the CTL line in response to LC-1 tumor cells (Fig. 1*C*). Furthermore, this CTL line failed to show significant levels of cytotoxicity to HLA-A24$^+$ tumor cells in the ^{51}Cr-release assay (Fig. 1*B*).

Among the eight peptides tested, only the peptides IEX47–56, IEX61–69, and IEX65–73 were recognized by the 850B-CTL cell line. All eight peptides tested had similar binding activities toward HLA-A33 molecules; thus, this result cannot be explained simply by differences in the binding affinities between the peptides and HLA-A33 molecules. Only the three peptides that were recognized by 850B-CTLs can be generated through the natural antigen-processing machinery *in vivo* and expressed in complex with HLA-A33 molecules on the cell surfaces of antigen presenting cells or tumor cells. Indeed, each of the three peptides induced peptide-specific CTLs in the PBMCs of HLA-A33$^+$ cancer patients in most of the cancer patients but in none of the healthy donors tested. This may be explained by the different frequencies of specific CTL precursors reacting to the peptides in the circulation of cancer patients (10, 11). This discrepancy could be explained by the fact that the IEX protein is preferentially expressed in cancer cells but not in normal epithelial cells or cells of connective tissues. IEX47–56 induced peptide-specific CTLs from all four of the patients tested, whereas the other two peptides induced CTLs in three of the four patients. This may be partly due to a difference in precursor frequencies of each peptide-specific CTL in the circulation of each cancer patient. The profiles of peptide-specific CTLs could be different from patient to patient.

HLA-A33 is one of the most common HLA-A alleles in Asians and blacks and is found in 13% of Japanese, 14% of Koreans, 4% of Caucasians, and 16% of blacks (14, 15). The three IEX-1-derived peptides induced HLA-A33-restricted, tumor-specific CTLs in the PBMCs of patients with GC and other epithelial cancers. Because IEX-1 is highly expressed in cancer tissues, these peptides might be appropriate target molecules for specific immunotherapy for HLA-A33$^+$ cancer patients.

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Immunological evaluation of vaccination with pre-designated peptides frequently selected as vaccine candidates in an individualized peptide vaccination regimen

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Abstract. We previously conducted a trial of an 'individualized' regimen, in which cancer patients were vaccinated with peptides after the confirmation of pre-existing peptide-specific cytotoxic T lymphocyte (CTL) precursors. In this study, we performed a new trial of 'pre-designated' regimen, in which cancer patients were vaccinated with peptides that were frequently selected as vaccine candidates in the preceding individualized regimen. Eighteen cancer patients (10 with uterine cervical cancer and 8 with gastric cancer) were enrolled in the new regimen. The pre-designated regimen was well tolerated by all patients. Although peptide-specific CTL precursors and humoral responses increased in the majority of patients with the pre-designated regimen, neither of the responses correlated with clinical outcome. Three patients had long stable disease, and their pre-vaccination peripheral blood mononuclear cells contained peptide-specific CTL precursors reactive to more than 2 of 4 peptides. With the pre-designated regimen, the levels of pre-existing immunoglobulin G reactive to non-vaccinated peptides decreased in 5 of 15 patients with progressive disease, and their time to progression was very short, whereas such a decrease was rarely observed in the preceding individualized regimen. These results suggest that the pre-designated regimen can elicit a primary immune response, but may incidentally suppress pre-existing immune responses.

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Abbreviations: CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; GC, gastric cancer; IFN, interferon; Ig, immunoglobulin; OD, optical density; PBMC, peripheral blood mononuclear cell

Key words: cancer vaccine, peptide, cytotoxic T lymphocyte, antibody

Introduction

We previously reported a panel of antigenic peptides capable of inducing tumor-reactive cytotoxic T lymphocytes (CTLs) in HLA-A24$^+$ or HLA-A2$^+$ patients (1-5). Subsequently, we conducted clinical trials of peptide vaccination using two different regimens (6-12). In the first regimen, lung, colon, and cervical cancer patients were vaccinated with CypB-, SART3-, and SART2-derived peptides, respectively (6,7,12). The vaccination protocols were completely safe, however, neither objective response nor immune responses were observed. In the second regimen, named an 'individualized' vaccination, HLA-A24$^+$ or HLA-A2$^+$ patients with advanced epithelial cancers were vaccinated with peptides for which the existence of CTL precursors was confirmed in the pre-vaccination peripheral blood mononuclear cells (PBMCs). Increased cellular and humoral immune responses to the vaccinated peptides were observed at higher frequencies in the post-vaccination PBMCs and sera, respectively (8-12). Furthermore, 3 of 5 patients with uterine cervical cancer showed objective tumor regression (12), and 2 of 4 patients with scirrhous types of gastric cancer (GC) have been disease-free for more than 2 years (11).

The individualized vaccination was suggested to be therapeutically effective, but this regimen has one major disadvantage, namely the requirement of screening peptide-specific CTL precursors prior to vaccination. This requirement hampers its worldwide development as a standard treatment modality. Therefore, in this study, we performed a new trial of a 'pre-designated' regimen in which cancer patients were vaccinated with 4 peptides, all of which were frequently selected as vaccine candidates in the preceding individualized vaccine regimen (8-12). The results show that, although potent

immune responses to administered peptides could be induced by the pre-designated regimen, this protocol was suggested to incidentally suppress the pre-existing immune responses compared with the individualized regimen.

Materials and methods

Patients and eligibility criteria. The Institutional Ethics Review Boards of Kurume University, Hokkaido University, Kochi University, and Kyoundo Hospital approved these clinical protocols of pre-designated peptide vaccination. Written informed consent was obtained from all the patients at the time of enrollment. According to the protocol, patients were required to be positive for HLA-A24 or -A2 as determined by conventional serological typing of HLA-class I expression on PBMCs as reported previously (11,12). All patients were pathologically confirmed to have gastric adenocarcinoma or uterine cervical carcinoma. Eligibility criteria included an age of ≤80 years, serum creatinine of <1.4 mg/dl, bilirubin of <1.5 mg/ dl, platelet count of ≥100,000/μl, hemoglobin of ≥8.0 g/dl, and total white blood cells of ≥3,000/μl. Hepatitis B surface antigen and hepatitis C RNA were required to be negative. The patients were untreated for at least 4 weeks before entry to the study, and had to have an Eastern Cooperative Oncology Group performance status of 0 to 2 at the time of entry. Patients with evidence of serious illness, immunosuppression, or autoimmune disease were excluded. Treatment was carried out from August 2001 through June 2003.

Peptides and vaccination. The peptides used in the present study were prepared under the condition of Good Manufacturing Practice by Multiple Peptide Systems (San Diego, CA). The sequences of the peptides are as follows: peptides used for HLA-A24$^+$ patients were $SART3_{109}$ (VYDYNCH VDL), $SART3_{315}$ (AYIDFEMKI), lck_{208} (HYTNASDGL), lck_{488} (DYLRSVLEDF), and $SART2_{93}$ (DYSARWNEI). Those used for HLA-A2$^+$ patients were $CypB_{172}$ (VLEGMEVV), lck_{246} (KLVERLGAA), ppMAPkkk$_{432}$ (DLLSHAFFA), and $UBE2V_{43}$ (RLQEWCSVI). All of these peptides have demonstrated their ability to induce HLA-A24- or HLA-A2-restricted and tumor-specific CTL activity in the PBMCs of cancer patients (2-12). They were frequently administered to cancer patients in the preceding individualized vaccination regimen in which cellular responses to peptides had been confirmed prior to vaccination (8-12). Although all peptides for HLA-A2$^+$ patients were selected based on the binding motif to HLA-A*0201 molecules, these peptides are immunogenic not only in HLA-A*0201 patients but also in those with other HLA-A2 subtypes such as HLA-A*0206 or HLA-A*0207 (2,11,12). Each peptide was dissolved in a small amount of dimethylsulfoxide, and diluted with saline at a concentration of 4 mg/ml. Montanide ISA-51, an incomplete adjuvant, was manufactured by Seppeic, Inc. (Franklin Lakes, NJ). One ml each of the resulting 4 different peptide emulsions (2 mg/peptide/injection) was injected subcutaneously into independent sites of the lateral abdominal wall for gastric cancer (GC) patients, while it was injected subcutaneously into independent sites of the lateral thigh for uterine cervical cancer patients using a glass syringe as reported previously (11,12). The interval of vaccination was 1 week and a total of 6 injections were performed. Peptide vaccination was continued thereafter, under informed consent, to further evaluate adverse events, immunological responses, and clinical responses under the regimen of individualized vaccination as reported previously (11,12). To choose peptides for the individualized vaccination, PBMCs that were drawn at the time of the 6th vaccination were provided for CTL precursor analysis to each of 14 or 16 different peptides in HLA-A24$^+$ or HLA-A2$^+$ patients, respectively. As well, up to 4 peptides (3 mg/injection) for which CTL precursors were detected in these PBMCs were administered to each patient every 2 weeks as reported previously (11,12).

Immunological assays. Skin tests were performed by intradermal injection of 50 μg of each peptide using a tuberculin syringe with a 26-gauge needle. Saline was used as a negative control. Immediate- and delayed-type hypersensitivity (DTH) reactions were determined at 20 min and 24 h after the injection, respectively. At least 10 mm of induration or 20 mm of erythema read 24 h after injection was required to score the DTH skin test as positive. For measurement of cellular responses, 30 ml of peripheral blood was obtained pre- and post (6th)-vaccinations, and PBMCs were separated by means of Ficoll-Conray density gradient centrifugation then used for CTL precursor assay as reported previously (11,12). In brief, PBMCs ($10x10^4$ cells/well) were incubated with 10 μM of each peptide in four different wells of a 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl culture medium containing interleukin (IL)-2 (100 units/ml), as reported previously (12). On the 14th day, the cells from each well were independently harvested, washed, and tested for their ability to produce interferon (IFN)-γ in response to C1R-A2402 cells for HLA-A24$^+$ peptides or to T2 cells for HLA-A2$^+$ peptides which were pulsed with a corresponding peptide or a negative control peptide (HIV peptide, RYLRDQQLL for HLA-A24 and SLYNTVATL for HLA-A2) in duplicate assays as reported previously (10-12). After an 18-h incubation, the supernatant was collected for measurement of IFN-γ by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). A two-tailed Student's t-test was employed for statistical analysis.

The serum levels of peptide-specific immunoglobulin G (IgG) were measured by ELISA as previously reported (10-12). In brief, 100 μl/well of serum samples from pre- and post (6th)-vaccinations were diluted with 0.05% Tween 20-Block Ace, and were added to the peptide (20 μg/well)-immobilized plate. After a 2-h incubation at 37°C, the plate was washed and further incubated for another 2 h with a 1:1,000-dilluted rabbit anti-human IgG (γ-chain-specific, Dako, Glostrup, Denmark). The plate was washed, then 100 μl of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase-dextran polymer (EnVision, Dako) was added to each well, and the plate was incubated for 40 min. After washing, 100 μl/well of tetramethyl-benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by an addition of 1 M phosphoric acid. The optical density (OD) values of each sample were shown as OD units/ml. For determining the cut-off value, patients' sera were measured at the same time

for their reactivity to both corresponding peptides and an HIV peptide, taken as a negative control peptide. OD value of >0.02 (OD value in response to a corresponding peptide subtracted from that in response to an HIV peptide at a serum dilution of 1:100) was considered as positive of peptide-reactive IgG.

Evaluation of clinical response. All known sites of disease were evaluated by CT-scan or X-ray examination before and after every 3-6 vaccinations. Patients were assigned to a response category according to the response evaluation criteria for solid tumors, a revised version of the WHO criteria published in June 1999 in the WHO Handbook for reporting results of cancer treatment.

Results

Patient characteristics and adverse events. Eighteen patients (10 with recurrent uterine cervical cancer and 8 with advanced GC) were enrolled in this study. Detailed characteristics of these patients are shown in Table I. The median age of the patients was 50, ranging from 27 to 74. Ten and 8 patients were HLA-A24 and -A2 positive, respectively. The sites of recurrence of cervical cancers were the vaginal stump (n=4), lymph nodes (n=4), lung (n=1), and vulva skin (n=1). The histology of cervical cancers was squamous cell carcinoma (n=8) or adenocarcinoma (n=2). Preceding treatments included surgical resection (n=7), chemotherapy (n=6), and radiotherapy (n=10). Five, 2, and 1 GC cases were stages IV, IIIB, and IIIA based on the Japanese Classification of GC, 13th edition, respectively. The histology of GC was all adenocarcinoma, in 6 cases (GC-3 to -8) scirrhous (diffuse) type and 2 cases non-scirrhous type. All GC patients underwent surgical resection of the primary lesion, and 6 of them had previously undergone failed chemotherapy.

All 18 patients were evaluated for adverse events. The vaccinations were generally well tolerated. Grade I and II fever with mild flu-like symptoms were observed in 4 patients and 1 patient, respectively; the fever was transient and self-limiting. Grade I and II local redness and swelling at the injection sites were observed in 12 and 4 patients, respectively. Nausea, vomiting, abdominal pain, and diarrhea were observed in 2 patients each, respectively. No severe (grade III or IV) toxicity was associated with the peptide vaccination. Grade I hematuria by bladder invasion and vaginal bleeding by vaginal stump were observed in one case each, while grade IV of vomiting of blood caused by tumor progression was observed in 1 GC patient (GC-7). There was no clinical evidence of an autoimmune reaction as determined by symptoms, physical examination, or laboratory tests.

Cellular immune responses. Cellular immune responses to the administered peptides were evaluated (Table II). To evaluate the cellular immune responses to the vaccinated peptides, both the pre- and post (6th)-vaccination PBMCs were thawed at the same time in order to avoid any *in vitro* biases during the assay of peptide-specific CTL precursors. The pre-vaccination PBMCs from the two cases (GC-1 and GC-3) were not available for analysis because of the limited numbers of cells, and the post-vaccination PBMCs from 3 cases (C-3, GC-5, and GC-7) were not available because of rapid progression and death. The assay of peptide-specific CTL precursors was performed in quadruplicate, and the amount of IFN-γ in response to an HIV peptide was subtracted. The results were assessed in terms of two parameters, the p-value and IFN-γ production, as described in the Table II legend. Peptide-specific CTL precursors were induced in 9 of 13 patients (69%). Four representative cases of cervical cancer (C-1, -7, -9, and -10) and 2 GC cases (GC-4 and -8) are shown in Fig. 1. For example, in one HLA-A24$^+$ GC patient (GC-4), none of the pre-vaccination PBMCs responded to any of the 4 vaccinated peptides, while each single well of the post-vaccination PBMCs responded to the $SART3_{109}$ peptide, the $SART3_{315}$ peptide, or the lck_{488} peptide. In an HLA-A2$^+$ cervical cancer patient (C-9), one well of the pre-vaccination PBMCs responded to the $UBE2V_{43}$ peptide, 2 wells responded to the lck_{246} peptide, and 2 wells responded to the $ppMAPkkk_{432}$ peptide. On the other hand, 4, 4, and 3 wells of the post-vaccination PBMCs responded to the $UBE2V_{43}$ peptide, the lck_{246} peptide, and the $ppMAPkkk_{432}$ peptide, respectively. These results indicate that the pre-designated regimen has the potential to effectively induce peptide-specific CTLs.

Humoral immune responses. We recently reported that the induction of IgG reactive to administered peptides correlates well with the clinical responses and outcomes of patients with several types of cancer (8,10,11). Therefore, we examined the levels of peptide-specific IgG kinetically. As shown in Table II, IgGs reactive to the peptides were detected in the pre-vaccination, and peptide-specific IgGs increased after the peptide vaccination (C-4, GC-1, -4, and -6). In addition, IgGs reactive to the administered peptides were induced in post-vaccination sera from 6 of 15 patients tested (C-1, -4, -6, -7, -8, and -10). In total, vaccine-induced induction or augmentation of peptide-specific IgG was observed in the post-vaccination sera of 9 of 15 patients tested (60%). The results of 9 cases whose sera had significant levels of IgG reactive to at least 1 peptide are shown in Fig. 2. In one HLA-A2$^+$ GC patient (GC-6), significant levels (<0.02 OD value at a serum dilution of 1:100) of IgG specific to the $UBE2V_{43}$ and lck_{246} peptides, but not to the other 2 peptides, were detectable in the pre-vaccination sera of this patient, and the peptide vaccination increased the level of peptide-specific IgG.

DTH. No delayed-type hypersensitivity (DTH) reaction to the peptides was observed before the vaccination in any of the patients tested. Peptide-specific DTH reactions were induced in 4 cervical cancer patients (C-1, -2, -6, and -7) until the 6th vaccination; the details of these reactions are given in Table II. For example, in patient C-1, DTH reactions to lck_{208} and lck_{488} peptides were observed after the 3rd and 4th vaccinations, respectively.

Clinical responses. The clinical responses are summarized in Table II. Major tumor regression (>50% reduction) was not observed in any of the patients. Four patients (C-3, GC-3, -5, and -7) had rapid progression and died due to cancer before the 6th vaccination. At the time of the 6th vaccination, among the 14 patients eligible for evaluation, 8 cases were diagnosed

Table I. Patient characteristics.

Case[a]	Age/ sex	HLA type	PS[b]	TNM (stage)[c]	Histology[d]	Site of metastases[e]	Previous treatments			No. of vaccination
							Surgery[f]	Chemotherapy[g]	Radiotherapy	
C-1	34/F	A24	0	T1b1N0M0 (IA) reccurent case	adeno	Vaginal stump	RH	CDDP/CPT11	Whole pelvis	13
C-2	46/F	A24	0	T4aN1M1 (IVA) reccurent case	adeno	Vaginal stump	TH	CDDP/CPT11	Whole pelvis	15
C-3	54/F	A24	0	T2bN1M0 (IIB) reccurent case	SCC	Paraortic LNs, virchow LNs, lung	RH	CDDP/PEP	Small pelvis	2
C-4	74/F	A24	0	T3bN0M0 (IIIB) reccurent case	SCC	Vaginal stump	-	-	Whole pelvis	12
C-5	52/F	A24	1	T2bN1M0 (IIB) reccurent case	SCC	Lung	RH	-	Whole pelvis	12
C-6	59/F	A2	0	T1b1N1M0 (IB) reccurent case	SCC	Paraortic LNs	RH	-	Whole pelvis	12
C-7	48/F	A2	0	T3bN1M0 (IIIB) reccurent case	SCC	Vulva skin	-	BOMP	Whole pelvis	13
C-8	27/F	A2	1	T2bN1M0 (IIB) reccurent case	SCC	Vaginal stump	RH	CDDP/CPT11 Taxol/CBDCA	Small pelvis	11
C-9	50/F	A2	0	T3bN1M0 (IIIB) reccurent case	SCC	Paraortic LNs, bone	-	CDDP	Whole pelvis	14
C-10	59/F	A2	0	T2bN1M0 (IIB) reccurent case	SCC	Pelvic LNs	RH	-	Whole pelvis	19
GC-1	74/M	A24	0	T3N2H0P0M0 (IIIB) reccurent case	adeno	Abdominal lymph nodes	DG	5FU, CDDP, 5'DFUR, UFT, TS-1, TXN	-	15
GC-2	68/M	A24	2	T3NxH0P1M0 (IV)	adeno	Carcinomatosis peritonitis	TG + S	FP, TS-1, UFT	-	8
GC-3	28/F	A24	1	T3NxH1P1M0 (IV)	adeno	Abdominal dessemination, liver, paraortic lymph nodes	BSO	TS-1	-	7
GC-4	50/F	A24	1	T3NxP1H0M0 (IV)	adeno	Abdominal dessemination	TG	-	-	9
GC-5	74/F	A24	1	T3NxH1P1M1 (IV)	adeno	Abdominal lymph nodes, lung	TG	-	-	3
GC-6	56/M	A2	1	T3N1H0P1M0 (IV)	adeno	Abdominal dessemination, carcinomatosis pleuritis	TG	TS-1	-	8
GC-7	45/M	A2	2	T3N2P0H0M0 (IIIB) reccurent case	adeno	Abdominal dessemination, paraortic lymph nodes	TG + S	Low dose FP MTX + LV	-	4
GC-8	50/M	A2	0	T3N1H0P0M0 (IIIA)	adeno	Abdominal dessemination	TG	5FU, TS-1, CDDP	-	6

[a]C, cervical cancer; GC, gastric cancer. [b]Performance status by ECOG score. [c]Japanese Classification of Gastric Carcinoma, the 13th edition, and, UICC ind FIGO Classification of Cervical Cancer (1997). [d]SCC, squamous cell carcinoma; adeno, adenocarcinoma. [e]LNs, lymph nodes. [f]RH, radical hysterectomy; TH, total hysterectomy; DG, distal gastrectomy; TG, total gastrectomy, BSO, bilateral salphingo oophorectomy. [g]CDDP, cisplatin; CPT11, irinotecan hydrochloride; BOMP, bleomycin hydrochloride + vincristine sulfate + mitomycin C + cisplatin; CBDCA, carboplatin; TS-1, 1 M tegafur-0.4 M 5-chloro2,4-dihydroxypyridine-1 M potassium oxonate; 5'DFUR, doxifluridine; 5FU, fluorouracil; LV, calcium leucovorin; MTX, methotrexate.

Table II. Summary of responses to the peptide vaccination.

Case	Peptide	Peptide-specific CTLp[a]		Antibody to peptide[b]		DTH[c] (post)	Clinical response after[d]		TTP[e] (days)	OS[f] (months)
		Pre	Post 6th	Pre	Post 6th		6th	12th		
C-1	$SART3_{109}$	-	C	-	+	-	SD	PD	152	10+
	$SART3_{315}$	-	C	-	-	-				
	lck_{208}	-	A	-	-	+ (3)				
	lck_{488}	A	Ar	-	-	+ (4)				
C-2	$SART3_{109}$	D	-	-	-	+ (5)	SD	PD	126	9+
	$SART3_{315}$	-	A	-	-	-				
	lck_{208}	-	-	-	-	+ (5)				
	lck_{488}	-	CC	-	-	-				
C-3	$SART3_{109}$	-	NT	-	NT	-	Dead	-	20	1
	$SART3_{315}$	-	NT	-	NT	-				
	lck_{208}	-	NT	-	NT	-				
	lck_{488}	-	NT	-	NT	-				
C-4	$SART3_{109}$	C	-	+	+	-	SD	SD	-	5+
	$SART3_{315}$	-	-	-	+	-				
	lck_{208}	-	-	-	+	-				
	lck_{488}	A	-	-	-	-				
C-5	$SART3_{109}$	-	-	-	-	-	PD	Dead	41	5
	$SART3_{315}$	-	-	-	-	-				
	lck_{208}	-	-	-	-	-				
	lck_{488}	AC	B	-	-	-				
C-6	$CypB_{172}$	-	-	-	+	-	PD	PD	43	15+
	lck_{246}	-	A	-	+	-				
	$ppMAPkkk_{432}$	-	CCCC	-	-	-				
	$UBE2V_{43}$	Ar	AA	-	+	+ (4)				
C-7	$CypB_{172}$	-	-	-	-	-	PD	Dead	65	8
	lck_{246}	-	AA	-	+	-				
	$ppMAPkkk_{432}$	-	C	-	-	-				
	$UBE2V_{43}$	-	AC	-	+	+ (3)				
C-8	$CypB_{172}$	-	A	-	-	-	PD	Dead	39	6
	lck_{246}	A	-	-	-	-				
	$ppMAPkkk_{432}$	-	-	-	-	-				
	$UBE2V_{43}$	-	-	-	+	-				
C-9	$CypB_{172}$	-	-	-	-	-	SD	SD	-	7+
	lck_{246}	CC	AAAB	-	-	-				
	$ppMAPkkk_{432}$	AC	AAB	-	-	-				
	$UBE2V_{43}$	A	ArAAA	-	-	-				
C-10	$CypB_{172}$	-	-	-	+	-	SD	PD	135	12+
	lck_{246}	-	-	-	+	-				
	$ppMAPkkk_{432}$	Ar	-	-	-	-				
	$UBE2V_{43}$	-	ArArC	-	+	-				
GC-1	$SART2_{93}$	NT[g]	ArC	-	-	-	SD	PD	89	5
	$SART3_{109}$	NT	AA	+	+	-				
	$SART3_{315}$	NT	A	-	-	-				
	lck_{488}	NT	A	-	-	-				

Table II. Continued.

Case	Peptide	Peptide-specific CTLp[a]		Antibody to peptide[b]		DTH[c] (post)	Clinical response after[d]		TTP[e] (days)	OS[f] (months)
		Pre	Post 6th	Pre	Post 6th		6th	12th		
GC-2	$SART2_{93}$	-	-	-	-	-				
	$SART3_{109}$	-	-	-	-	-	SD	Dead	53	2
	$SART3_{315}$	-	-	-	-	-				
	lck_{488}	A	Ar	-	-	-				
GC-3	$SART2_{93}$	NT	-	-	-	-				
	$SART3_{109}$	NT	-	-	-	-	Dead	-	43	2
	$SART3_{315}$	NT	C	-	-	-				
	lck_{488}	NT	-	-	-	-				
GC-4	$SART2_{93}$	-	-	-	-	-				
	$SART3_{109}$	-	A	+	+	-	PD	Dead	27	2
	$SART3_{315}$	-	C	-	-	-				
	lck_{488}	-	A	-	-	-				
GC-5	$SART2_{93}$	A	NT	-	NT	-				
	$SART3_{109}$	-	NT	-	NT	-	Dead	-	14	1
	$SART3_{315}$	-	NT	-	NT	-				
	lck_{488}	-	NT	-	NT	-				
GC-6	$CypB_{172}$	-	B	-	-	-				
	lck_{246}	-	AB	+	+	-	PD	Dead	47	2
	$ppMAPkkk_{432}$	-	B	-	-	-				
	$UBE2V_{43}$	-	-	+	+	-				
GC-7	$CypB_{172}$	-	NT	-	NT	-				
	lck_{246}	-	NT	-	NT	-	Dead	-	17	1
	$ppMAPkkk_{432}$	C	NT	-	NT	-				
	$UBE2V_{43}$	-	NT	+	NT	-				
GC-8	$CypB_{172}$	-	-	-	-	-				
	lck_{246}	-	ArArArAr	-	-	-	SD	SD	-	11+
	$ppMAPkkk_{432}$	D	B	-	-	-				
	$UBE2V_{43}$	BD	ABB	-	-	-				

[a]The CTL precursor induction assay was performed in quadruplicate, and the background response to the HIV peptide was subtracted from the value. The result was evaluated by the following criteria: Ar (p-value ≤0.1 and IFN-γ production ≥500 pg/ml), A (p≤0.05 and IFN-γ production >50), B (p<0.05 and 25< IFN-γ production <50), C (0.05<p<0.1 and IFN-γ production >50), and D (0.05<p<0.1 and 25< IFN-γ production <50). The classification is shown by letters of the alphabet, and each character represents the results of each well. For example, AAB means that 3 wells were judged as A, A, and B, and 1 well was negative. [b]The net OD value of >0.02 (OD value in response to a corresponding peptide subtracted from that in response to an HIV peptide at a serum dilution of 100) was considered as positive of peptide-reactive IgG. [c]Number of the vaccination when DTH to the peptide was detected for the first time. [d]SD, stable disease; PD, progressive disease. [e]TTP, time to progression. [f]OS, overall survival. Pulse (+) mark; patients are alive (9/15/2003). [g]NT, not tested.

with stable disease (SD) and the remaining 6 patients were diagnosed with progressive disease (PD). All of these 14 patients decided for themselves to enter into the individualized vaccination regime based on the screening of CTL precursors to peptides with the post (6th)-vaccination PBMCs. At the time of the 12th vaccination, among the 8 patients eligible for evaluation, 3 cases (C-4, -9, and GC-8) were diagnosed with SD, and the remaining 5 patients were diagnosed with PD. Tumor regression by means of a 16% size reduction of a paraortic lymph node was observed at the 10th vaccination in one case (C-9). The median observation time, median time to progression (TTP), and median survival time (MST) of 18 patients were 11, 1.5 (47 days), and 5 months (156 days), respectively.

Analysis of the 3 SD patients may aid in the evaluation of prognostic factors. In patient C-4, although peptide-specific IgG was induced, peptide-specific CTLs and DTH were not observed after the peptide vaccination. In patients C-9 and CG-8, although peptide-specific CTLs were induced, peptide-specific IgG and DTH were not observed. In comparison to the other 15 patients, one common finding among these 3 SD patients was that peptide-specific CTL precursors to more