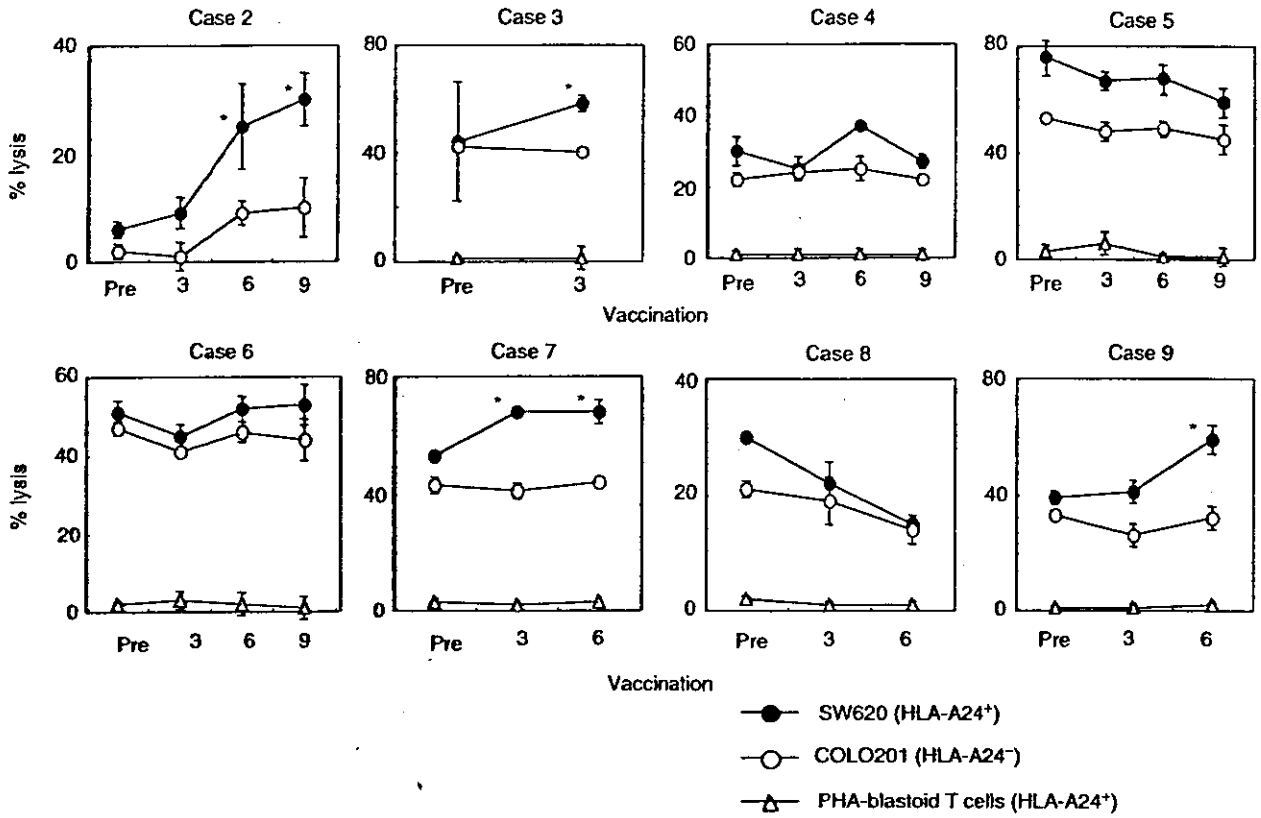

Figure 2 Cytotoxicity before and after the peptide vaccination. Pre- and post-vaccination PBMCs from eight patients were incubated for 14 days with IL-2 alone without any peptides in culture, followed by measurement of cytotoxicity against SW620 cells (HLA-A24$^+$ colon cancer cell line), COLO201 cells (HLA-A24$^-$ colon cancer cell line), and PHA-activated T cells (HLA-A24$^+$) by a 6-h ^{51}Cr-release assay at an *E/T* ratio of 40/1. The assay was performed in triplicate, and the mean and s.d. are shown. *Statistically significant at $P<0.05$.

in three patients after the peptide vaccination (Table 4). In patient 1, DTH reactions to lck_{208} and lck_{488} were observed after the 6th vaccination, and DTH against $SART3_{109}$ became detectable after the 9th vaccination. Patient 4 exhibited a DTH reaction against lck_{208}, lck_{486}, and lck_{488} after the 3rd vaccination. Patient 7 exhibited a DTH reaction against $SART3_{109}$ after the 3rd vaccination.

Clinical results

The clinical responses of each patient are summarised in Table 4. CT scans of patient 1 pre- and post-vaccination are shown in Figure 4A. In all, 48% regression (27–14 mm) of liver metastasis was observed in patient 1. This patient had para-aortic lymph node metastasis at a diameter of less than 20 mm; it showed no change after the peptide vaccination (data not shown). Because his clinical response was considered a partial response (PR), this patient was analysed more in detail. The kinetic analysis of tumour cell lysis in patient 1 indicates that increased CTL activity to the SW620 cells became detectable in post-vaccination PBMCs (Figure 4B). The cytotoxicity against lck_{208} peptide-loaded CIR-A2402 cells became significant after the 3rd and 6th vaccinations (Figure 4C). While no peptide-specific IFN-γ production was detected in any of the 96 wells containing 100 cells well^{-1} of pre-vaccination PBMCs, $SART3_{109}$-specific IFN-γ production was detected in two, two, and four wells among 96 wells containing post-vaccination (3rd, 6th, and 9th) PBMCs, respectively (Figure 4D). Production of lck_{208}-specific IFN-γ production was detectable in three and four wells among 96 wells containing the 6th and 9th vaccination PBMCs, while lck_{488}-specific IFN-γ production could be observed in one and two wells among 96 wells containing the 6th- and 9th-vaccination PBMCs, respectively. The patient has subsequently been treated only by vaccination ($SART3_{109}$, lck_{208}, and lck_{488}) for 7 months as an outpatient, and is still doing well. Patient 2 had intrapelvic metastasis, and the disease has remained stable (s.d.) for 6 months. The eight other patients showed progressive disease (PD) 2–4 months after starting the vaccinations, although all have been treated as outpatients and their quality of life has been evaluated as quite high.

DISCUSSION

Patients undergoing this regimen received 3 mg of peptides biweekly for up to four peptides. All of the peptides used were derived from nonmutated self-antigens involved in cellular proliferation (Kikuchi *et al*, 1999; Gomi *et al*, 1999; Yang *et al*, 1999; Harashima *et al*, 2000; Kawano *et al*, 2000; Nakao *et al*, 2000; Nishizaka *et al*, 2000). However, there was no grade III or IV adverse effect, which is consistent with previous observations in studies of peptide-based vaccinations. Most of the patients received the vaccination as outpatients, and the performance status remained very good throughout the treatment periods. Therefore, in terms of safety, this regimen of CTL precursor-oriented peptide vaccine could be recommended as a cancer vaccine suitable for further clinical trials.

Another aim of our study was to assess the clinical response to the vaccination. In our limited number of case, we observed one PR and one s.d. continuing for more than 6 months. Both of these cases were treated with three kinds of SART3- and lck-derived

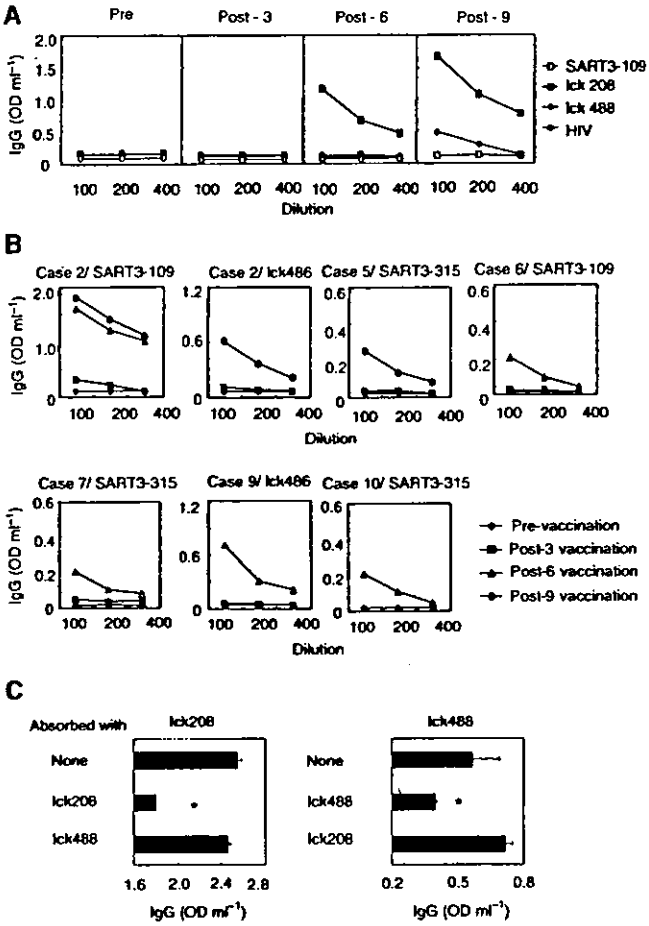

Figure 3 IgG reactive to the vaccinated peptides. (**A**) Pre- and post-vaccination sera from patient 1 were serially diluted and the levels of IgGs reactive to three administered peptides were determined by ELISA. (**B**) Pre- and post-vaccination sera from patients 2, 5, 6, 7, 9, and 10 were serially diluted and the levels of IgGs reactive to corresponding peptides were determined by ELISA. (**C**) Diluted sera of patient 1 after the 9th vaccination were cultured in the indicated peptide-coated wells and the levels of IgGs reactive to corresponding peptides were determined by ELISA. *Statistically significant at $P<0.05$.

peptides, suggesting that the combined use of these peptides might constitute a promising vaccine strategy for advanced colorectal carcinomas, thus encouraging us to plan a phase II trial utilising these peptides.

Vaccination-induced immunity was evaluated in this study by several different methods, including IFN-γ production in response to peptides, a standard 6-h ^{51}Cr-release assay, measurement of antipeptide antibody, and DTH responses. An elevated immune response to lck_{208} and lck_{488} was detected in post-vaccination PBMCs by all of the methods used in the samples of patient 1, who showed PR. This patient's PBMCs also reacted to the $SART3_{109}$ peptide, as measured by frequency analysis of cellular responses to peptides (Figure 4D) and also by DTH test (Table 4). These results indicate that the patient's PBMCs reacted to all three vaccinated peptides after the peptide vaccination. Post-vaccination PBMCs from patient 2, who had a long s.d., responded to lck_{208} peptide alone, and the post-vaccination sera became positive for both the $SART3_{109}$ and lck_{486} peptides, although no DTH response was observed (Table 4). Besides patient 1, positive DTH response was observed in only two patients (4 and 7), with PD, but their post-vaccination PBMCs showed no increase in cellular responses to the administered peptides. On the other hand, besides patients 1 and 2, IgG reactive to the administered peptides became detectable in the post-vaccination sera of five other patients (5, 6, 7, 9, and 10) with PD. Although the post-vaccination PBMCs of patients 6 and 10 showed an increase in cellular responses to $SART3_{109}$ and $SART3_{315}$, respectively, no augmentation of peptide-specific cellular response was observed in other cases. Neither a cellular nor humoral immune response to administered peptides was detectable in the remaining two patients (3 and 8), who also had PD. These results suggest that vaccination-induced immunity varies considerably among patients. However, we recently reported that the *in vivo* induction of IgG reactive to administered peptides is positively correlated with clinical response or the survival of patients with prostate, lung, gastric, or gynaecological cancer (Mine *et al*, 2003; Noguchi *et al*, 2003; Sato *et al*, 2003; Tsuda *et al*, 2004). This may be the case with patients 1 and 2, who showed PR and s.d., respectively, because IgGs reactive to two different peptides were induced only in these two patients. We have no clear answer regarding the role of peptide-specific IgG in antitumour immune response, and are now grappling with this theme.

In addressing the mechanism for peptide-specific IgG induction after peptide vaccination, one possibility is that 9-mer or 10-mer peptide-recognizing $CD4^+$ T cells were involved in this phenomenon. In general, *in vivo* generation of antigen-specific IgG requires a cytokine from helper T cells (Parker, 1993). Although peptides binding to MHC class II molecules have been suggested to be 12–25 amino acids in length, the core sites anchored to MHC class II molecules are sufficient even at a length of about nine amino acids (Rammensee *et al*, 1995). Indeed, we recently observed that peptide vaccination with a 9-mer peptide could induce peptide-specific and HLA-DR-restricted $CD4^+$ T cells *in vivo* (Harada *et al*, 2004). $CD4^+$ T-cell help is required during the generation and maintenance of effective anti-tumour $CD8^+$ T cell-mediated immunity. The requirement of $CD4^+$ T-cell help to initiate and sustain a $CD8^+$ T-cell response has been well established and has led to the development of antitumour vaccines that attempt to induce both T-cell subsets (Knutson *et al*, 2001). The *in vivo* induction of IgG reactive to administered peptides may be indirect evidence of the involvement of $CD4^+$ T lymphocytes.

We recently developed a culture system to evaluate CTL precursors against many peptides using a limited number of PBMCs from cancer patients (Hida *et al*, 2002); the same culture system was applied to this study. The main reason why we assessed peptide-specific CTL precursors based on two parameters, the *P*-value and IFN-γ production, was that the levels of IFN-γ produced by peptide-specific CTLs varied among quadruplicate wells. This finding might be due to the small number of cells (10^5 cells well^{-1}) that were initially placed in each well. It is possible that one well may have contained peptide-specific CTL precursors, whereas another may have contained none. We concluded that each well should be individually estimated to screen for the presence of peptide-specific CTL precursors.

Recent reports revealed that a Th2 response is predominant in cancer patients (Pellegrini *et al*, 1996; Vita *et al*, 1999; Sheu *et al*, 2001). Therefore, we examined the level of IL-4 during peptide stimulation *in vitro*, but the level of IL-4 production was generally low, and no peptide-specific IL-4 production was observed. In contrast, the level of IFN-γ production was constantly substantial. Probably, the *in vitro* culture of PBMCs in the presence of IL-2 could preferentially activate natural killer cells, and natural killer cell-derived IFN-γ might provide an optimal condition for Th1 type cells.

In conclusion, vaccination of colorectal cancer patients with peptides by the CTL precursor-oriented method was a well-tolerated outpatient treatment and induced antigen-specific immunity as well as a clinical response. Even though only a small number of selected patients were treated, the encouraging clinical response demands further studies of CTL precursor-oriented vaccine in other human cancers.

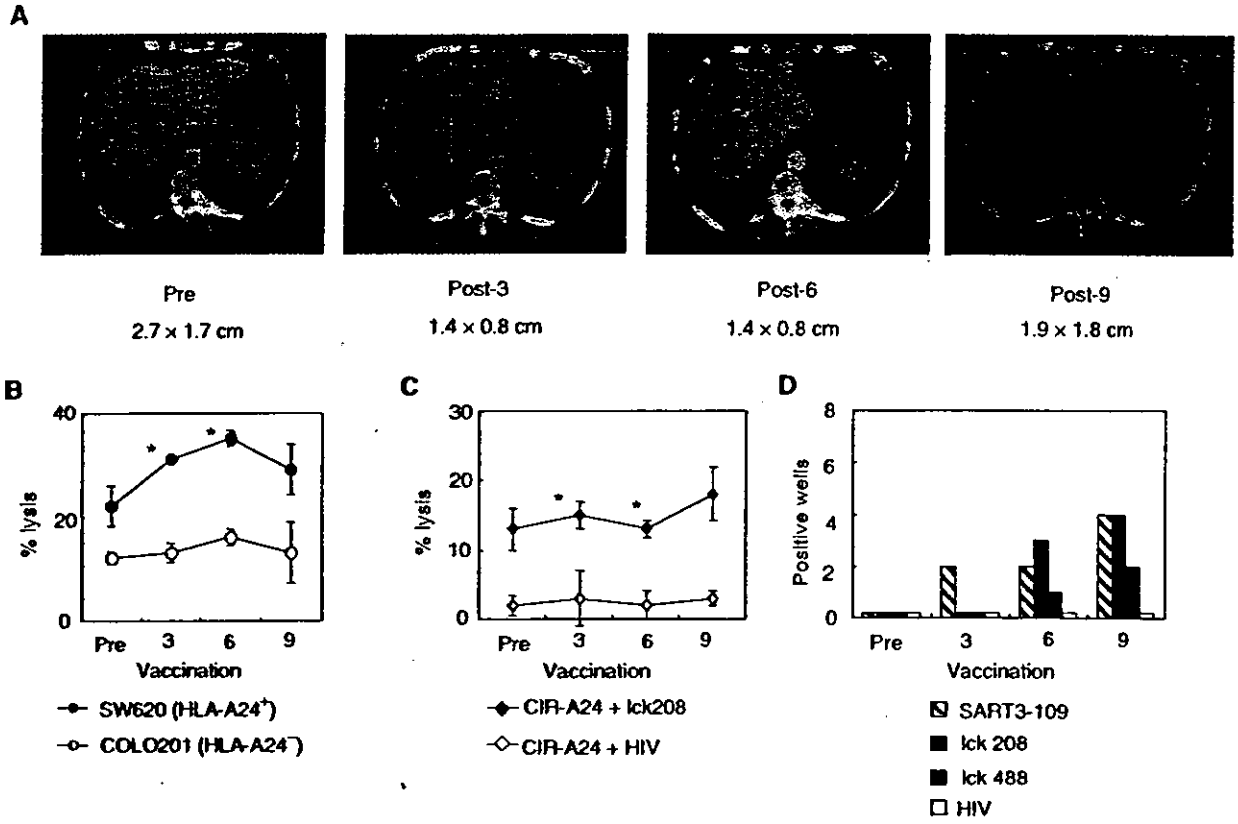

Figure 4 Clinical and immunological responses to the peptide vaccination. (**A**) CT scans show tumour regression of the liver metastasis after the peptide vaccination. The size of the liver metastasis (S8) is described. (**B**) CTL activity before and after vaccinations. Cytotoxicity to SW620 cells (HLA-A24$^+$ colon cancer cell line), COLO201 cells (HLA-A24$^-$ colon cancer cell line), and PHA-activated T cells (HLA-A24$^+$) was tested by a 6-h ^{51}Cr-release assay at an *E/T* ratio of 40/1. Values are the means of triplicate assay. *Statistically significant at $P<0.05$. (**C**) Peptide-specific CTL activity before and after the peptide vaccinations. Cytotoxicity to CIR-A2402 cells preloaded with the Ick_{208} or the control HIV peptide was tested by a 6-h ^{51}Cr-release assay at an *E/T* ratio of 40/1. *Statistically significant at $P<0.05$. (**D**) Kinetics of peptide-specific CTL precursors. Pre- and post- (3rd, 6th, and 9th) vaccination PBMCs were incubated at 100 cells per well in a 96-well round-microculture plate in the presence of feeder cells. The number of wells producing a significant level of IFN-γ in a peptide-specific manner among 96 wells is shown.

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Identification of epidermal growth factor receptor-derived peptides immunogenic for HLA-A2$^+$ cancer patients

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Epidermal growth factor receptor (EGFR) is one of the most appropriate target molecules for cancer therapy because of its relatively high expression in about one-third of all epithelial cancers in correlation with neoplasmic progression. With respect to EGFR-targeted therapies, antibodies and tyrosine-kinase inhibitors have been intensively studied, a novel EGFR-tyrosine-kinase inhibitor ZD1839 has been approved as an anticancer drug, and many other agents are now under clinical trial. In addition, cytotoxic T lymphocyte (CTL)-directed epitope peptides could be another class of compounds useful in EGFR-targeted therapies. However, there is presently no information on CTL-directed peptides of EGFR. Therefore, from the viewpoint of development of peptide-based cancer therapy, this study was intended to determine the EGFR-derived peptides recognised by both cellular and humoral immunities in HLA-A2$^+$ epithelial cancer patients. We herein report finding of two such types of EGFR-derived peptides at position 479–488 and 1138–1147, both of which were recognised by the majority of patients' sera (IgG), and also possessed the ability to induce HLA-A2-restricted peptide-specific CTLs against EGFR-positive tumour cells in peripheral blood mononuclear cells (PBMCs) of epithelial cancer patients. These results may provide a scientific basis for the development of EGFR-based immunotherapy for HLA-A2$^+$ cancer patients.

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Epithelial growth factor receptor (EGFR) plays an important role in epithelial biology and in many human malignancies (Coussens *et al*, 1985; Yamamoto *et al*, 1986; Salomon *et al*, 1995). A line of evidence that the EGFR plays a role in the pathogenesis of various cancers has led to the rational design and development of agents that selectively inhibit this receptor. Classes of compounds used in these EGFR-targeted therapies are mainly antibodies (Abs) and tyrosine-kinase inhibitors. Among them, ZD1839 (Iressa) is therapeutically effective for patients with advanced non-small-cell lung cancer (NSCLC) (Fukuoka *et al*, 2003; Miller *et al*, 2003). In addition, cytotoxic T lymphocyte (CTL)-directed epitopes could be another class of compound useful in EGFR-targeted therapies as peptide vaccines for cancer patients whose tumours overexpress EGFR. However, there is little information on CTL-directed epitopes of EGFR, although such CTL-directed peptides of HER2/neu, a family of EGFR, have been reported over the past decade (Fisk *et al*, 1995; Peoples *et al*, 1995; Kawashima *et al*, 1999; Okugawa *et al*, 2000). In previous clinical studies, we reported that some CTL-directed peptides from nonmutated proliferation-related proteins had the ability to elicit both cellular and humoral immune responses *in vivo* (Mine *et al*, 2003; Noguchi *et al*, 2003; Sato *et al*, 2003). Further, the levels of antipeptide Abs in postvaccination sera were well correlated with the overall survival of advanced lung cancer patients who received peptide vaccination (Mine *et al*, 2003). In addition, there is a line of evidence suggesting the existence of more highly immunogenic peptides that are capable of inducing both cellular and humoral immune responses (Disis *et al*, 1997). Therefore, to assist in the development of peptide-based cancer therapy, we here attempted to identify such peptides, and report the discovery of two peptides that can be considered as vaccine candidates for HLA-A2$^+$ cancer patients.

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MATERIALS AND METHODS

Samples and cell lines

After written informed consent was obtained, sera and peripheral blood mononuclear cells (PBMCs) were collected from NSCLC patients at Kurume University Hospital. Peripheral blood mononuclear cells and sera were also obtained from healthy donors (HDs). All subjects were free from human immunodeficiency virus (HIV) infection. All sera and PBMCs were cryopreserved at −80 and −196°C until use, respectively. The expression of HLA-class I antigens on these PBMCs was serologically defined by the conventional methods as reported previously (Noguchi *et al*, 2003), and HLA-A2 subtypes were determined by the sequence-specific oligonucleotide probe method as reported previously (Ito *et al*, 2001). The following tumour cell lines were used as target cells in a 6-h ^{51}Cr-release assay in this study: 11–18 (HLA-A2/24, human lung adenocarcinoma, EGFR$^+$), QG56 (HLA-A26, human lung squamous cell carcinoma, EGFR$^+$), SKOV3 (HLA-A3/28,

human ovarian cancer, EGFR$^+$) and SKOV3-A2 (HLA-A2-transfected SKOV3). The expression of EGFR in these cell lines except 11-18 was already reported (Xu *et al*, 1999; Hasmann *et al*, 2003). The expression of EGFR in 11-18 tumour cells was checked by flow cytometric assay with an immunofluorescence-labelled anti-EGFR monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Parkar *et al*, 2001), and it was also expressed in 11-18 tumour cells (data not shown). Phytohaemagglutinin (PHA)-blastoid T cells from PBMCs were also used as a negative control of target cells for a 6-h ^{51}Cr-release assay. For peptide loading, T2 (HLA-A2, T-B hybridoma) cells were also used in this study.

Peptides and quantification of antipeptide-specific IgG

The following peptides were purchased from BioSynthesis (Lewisville, TX, USA): 29 kinds of EGFR-derived peptides with HLA-A0201 and A0205 binding motifs at positions 10-18, 40-49, 61-70, 88-96, 110-118, 431-440, 479-488, 599-607, 653-662, 654-662, 656-664, 665-674, 681-689, 702-800, 717-725, 729-738, 765-776, 777-786, 791-799, 811-819, 813-822, 813-821, 843-851, 852-861, 944-952, 944-953, 945-953, 1001-1010, and 1138-1147, respectively. An HIV peptide with an HLA-A0201 binding motif (SLYNTVATL) was also provided as a negative control. Antipeptide-specific IgG levels in sera were measured by an enzyme-linked immunosorbent assay (ELISA) as reported previously (Sato *et al*, 2003). In brief, serum samples were serially diluted with 0.05% Tween 20-Block Ace (Yukijirushi Nyugyo, Tokyo, Japan), and 100 μl well^{-1} of diluted serum was added to the peptide (20 μg well^{-1})-immobilized Nunc Covalink plates (Roskilde, Denmark). Antipeptide Abs were detected with a rabbit anti-human IgG (γ-chain-specific) (DAKO, Glostrup, Denmark). For determining the limit of sensitivity of ELISA, sera from 11 HDs (HIV-negative) were measured for their reactivity to an HIV peptide by the assays, and the mean $\pm$ 2 standard deviations (s.d.) of optimal density (OD) at a serum dilution of 1:100 was 0.02 $\pm$ 0.04. The mean + 2 s.d. value (0.06) was then determined as the cutoff value. To test the specificity of antipeptide IgG in serum samples, 100 μl well^{-1} of serum samples (100 times dilution with 0.05% Tween 20-Block Ace) was absorbed with immobilised peptides (20 μg well^{-1}) in wells of the plate for 2 h at 37°C. The absorption, followed by testing of the antipeptide IgG with ELISA, was repeated three times. To test the antipeptide IgG response to a whole molecule of EGFR, patients' sera possessing antipeptide activity were also absorbed with either immobilised human EGFR isolated from A431 cells with a purity of 85% (UPSTATE, Charlottesville, VA, USA) or immobilised human albumin as a negative control, followed by measurement of anti-peptide activity by ELISA.

To test the direct growth inhibition activity of antipeptide IgG, 11-18 tumour cells were cultured in the presence of three different concentrations of sera that had detectable levels of antipeptide activity. Namely, 11-18 cells at 1×10^3 cells well^{-1} in a 96-well microculture plate (IWAKI, Chiba, Japan) were cultured for 12 h in the medium with 10% FCS followed by replacement of the culture medium to serum-free RPMI1640 with 1, 2, or 5% of serum possessing antipeptide activity. As controls, these sera absorbed with a corresponding peptide, sera without antipeptide activity from the two patients and two HDs were used. A total of 11-18 cells were also cultured in the RPMI1640 with 1, 2 and 5% FCS, respectively. After 24, 48 and 72 h incubation, the number of viable cells was determined by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). We also tested the antibody-dependent cell-mediated cytotoxicity of antipeptide IgG. Namely, the cytotoxicity of freshly-isolated PBMCs from HLA-A2$^+$ HDs against T2 cell pulsed with an EGFR-derived peptide or an HIV peptide as a negative control was measured in the presence of heat-inactivated serum possessing antipeptide activity by a standard 6-h ^{51}Cr-release assay. As controls, these sera absorbed with a corresponding peptide, sera possessing nonpeptide activity, and serum-free RPMI1640 medium were used. All sera used in the cytotoxicity assay were heat-inactivated at 56°C for 30 min.

CTL induction

Peripheral blood mononuclear cells from HLA-A2$^+$ epithelial cancer patients and HDs served as samples for the CTL induction assay. For induction of peptide-specific CTLs, PBMCs (15×10^4 cells well^{-1}) were incubated with 10 μM of each peptide in four different wells of a 96-well microculture plate (Nunc) in 200 μl culture medium containing interleukin-2 (IL-2), as reported previously (Mine *et al*, 2003). On the 14th day, the cells from each well were independently harvested, washed, and tested for their ability to produce interferon-γ (IFN-γ) in response to T2 cells pulsed with a corresponding peptide or a negative control peptide (HIV) in the duplicate assays. After an 18-h incubation, the supernatant was collected and measured for IFN-γ production by ELISA. Then the cells in the wells producing IFN-γ in response to a corresponding peptide were collected and further cultured with IL-2 alone for 10-14 days to obtain a large number of cells for a standard 6-h ^{51}Cr-release assay against the various tumour cells described above. The method used for the ^{51}Cr-release assay has been reported elsewhere (Mine *et al*, 2003). For an inhibition test, we used 20 μg/ml of anti-HLA-class I (W6/32, IgG2a), anti-HLA-class II (H-DR-1, IgG2a), anti-CD4 (Nu-Th/i, IgG1), and anti-CD8 (Nu-Ts/c, IgG2a) mAbs. We also used an anti-CD14 (JML-H14, IgG2a) mAb as a negative control. For a competition assay to study the peptide specificity of the cytotoxicity, unlabelled T2 cells pulsed with the corresponding peptide or an HIV peptide as a negative control were added to the ^{51}Cr-release assay at a cold-to-hot target cell ratio of 10:1. A two-tailed Student's *t*-test was employed for the statistical analysis in this study.

RESULTS

We first investigated whether IgG reactive to each of the 29 different EGFR-derived peptides could be detected in the sera of 20 cancer patients and 11 HDs. Representative results are shown in Figure 1 and a summary of the results on 11 different peptides to which at least two of the sera showed a positive response is given in Table 1. Significant levels of IgG (>0.06 OD values at a serum dilution of 1:100) reactive to the $EGFR_{479-488}$, $EGFR_{729-738}$, and $EGFR_{1138-1147}$ peptides were detected in the sera of 13, 10, and 10 patients, respectively. Sera from 2, 0, and 5 out of 11 HDs tested also showed significant levels of IgG reactive to the $EGFR_{479-488}$, $EGFR_{729-738}$, and $EGFR_{1138-1147}$ peptides, respectively. In addition, the significant levels of IgG reactive to the $EGFR_{110-118}$, $EGFR_{599-607}$, and $EGFR_{765-776}$ peptides were detected in sera from each of four cancer patients as well as a few HDs. The IgG reactive to the other five peptides was also observed in the sera of several patients and HDs. These humoral responses to EGFR peptides were observed in both HLA-A2-positive and -negative subjects, indicating no apparent HLA-A2 restriction to peptide-reactive IgG as reported previously (Ohkouchi *et al*, 2002). In contrast, significant levels of IgG reactive to the remaining 21 peptides were not detectable in any of the sera tested (data not shown). Subsequently, we focused our efforts on the five peptides $EGFR_{110-118}$, $EGFR_{479-488}$, $EGFR_{729-738}$, $EGFR_{852-861}$, and $EGF\text{-}R_{1138-1147}$ (>90% purity) in the following study.

The peptide specificity of antipeptide IgG to each of the $EGFR_{110-118}$, $EGFR_{479-488}$, $EGFR_{729-738}$, $EGFR_{852-861}$, and $EGF\text{-}R_{1138-1147}$ peptides was confirmed by absorption tests. Representative results of the peptide specificity for each of the five peptides by means of the absorption tests are shown in Figure 2 in which the results on sera from two patients were provided for each peptide.

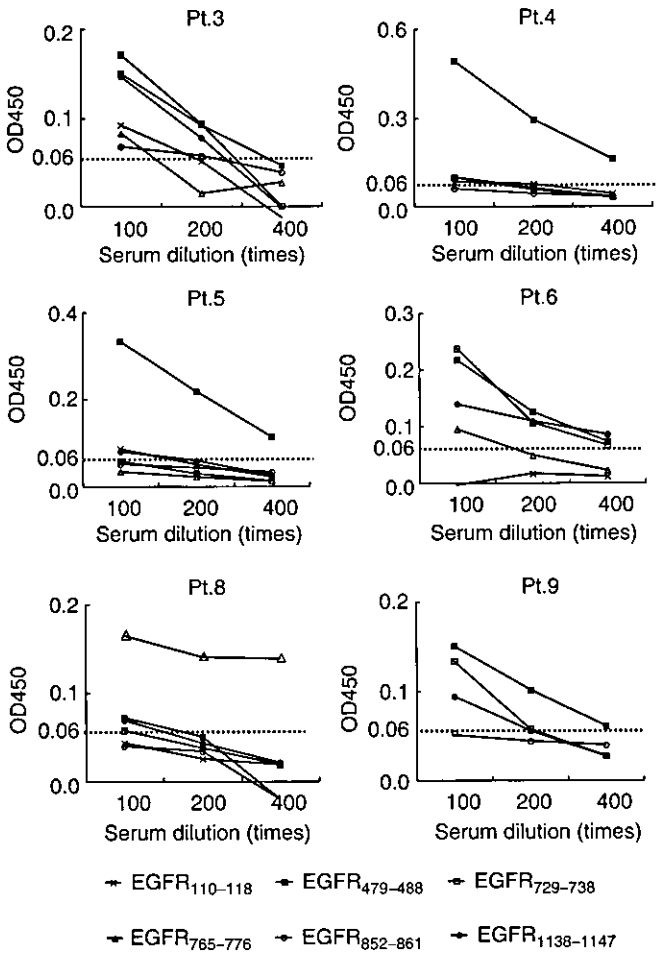

Figure 1 Detection of antipeptide IgG. Optical density values of each sample were assayed in serially diluted serum samples to estimate peptide-reactive IgG levels by the ELISA. The OD value against an irrelevant peptide (HIV) used as a negative control was subtracted from the data. Representative results of six patients (Pts.3, 4, 5, 6, 8, and 9) are shown. The cutoff value was set as 0.06 OD value at a serum dilution of 100 times (the mean (0.02) + 2 s.d. (0.02) of OD value in HDs ($n = 11$) in response to an HIV peptide which has an HLA-A2 binding motif taken as a negative control).

As can be seen, the activity of these sera reactive to each peptide was absorbed with a corresponding peptide, but not with an HIV peptide used as a negative control. We also investigated by an absorption test whether antipeptide IgG reacts to the whole EGFR protein. The level of the antipeptide IgG reactive to any of these peptides, however, was not decreased at all by the absorption test. Representative results of the two peptides ($EGFR_{479-488}$ and $EGFR_{1138-1147}$) are shown in the lower, right-hand columns of Figure 2. These results suggest that there was no crossreactivity between antipeptide IgGs and the whole EGFR protein.

Based on these findings, these $EGFR_{110-118}$, $EGFR_{479-488}$, $EGFR_{599-607}$, $EGFR_{729-738}$, $EGFR_{765-776}$, and $EGFR_{1138-1147}$ peptides were further tested for their abilities to induce CTL activity in PBMCs of HLA-$A2^+$ epithelial cancer patients and HDs ($n = 10$ and 6). The $EGFR_{813-822}$ peptide, to which no IgG response was detectable in sera, was also tested as a control. We judged the induction to be successful when the supernatant of at least one well showed more than 100 pg ml^{-1} INF-γ production with a statistically significant difference (P-value of <0.05). The $EGFR_{479-488}$ and $EGF\text{-}R_{1138-1147}$ peptides induced peptide-specific CTLs in three and six of 10 cancer patients tested, respectively. Representative results (Pts.3, 5, 6, 8, 9, and 10) are shown in Figure 3, in which the results from each of the four wells are provided. Background INF-γ productions in response to an HIV peptide (<50 pg ml^{-1}) were subtracted. In regard to HLA-A subtypes, two (Pts.5 and 9), three (Pts.3, 6, and 8), and one (Pt.10) patients were HLA-A0201, -A0206, and -A0207, respectively (Table 1). The results indicate that these two peptides had the ability to induce a peptide-specific cellular response in PBMCs from different HLA-A2 subtypes. These two peptides, however, were not sufficiently stimulated to produce significant levels of IFN-γ in any of the six HDs tested. Similarly, each of the other five peptides tested rarely stimulated PBMCs to produce the significant levels of IFN-γ in either cancer patients or HDs (data not shown).

Sera possessing anti- $EGFR_{479-488}$ and anti-$EGFR_{1138-1147}$ peptide activities from the patient 4 and patient 6 were tested for their capability to directly inhibit growth of 11–18 tumour cells. As controls, these sera absorbed with a corresponding peptide, sera without antipeptide activity from the two patients and two HDs, and FCS were used. However, none of the sera with antipeptide IgG directly inhibited tumour cell growth *in vitro*. Representative results at a serum concentration of 5% are shown in Figure 4 (left side). We also tested the antibody-dependent cell-mediated cytotoxicity of antipeptide IgG. Namely, the cytotoxicity of freshly isolated PBMCs from HLA-$A2^+$ HDs against T2 cell pulsed with an EGFR-derived peptide or an HIV peptide as a negative control was measured in the presence of heat-inactivated serum possessing antipeptide activity. As controls, these sera absorbed with a corresponding peptide, sera possessing nonpeptide activity, and serum-free RPMI1640 medium were used. However, the presence of sera possessing anti-EGFR peptide activity could not increase their cytotoxicity. Representative results are shown in Figure 4 (right side).

The cytotoxicity of the $EGFR_{479-488}$ or $EGF\text{-}R_{1138-1147}$ peptide-stimulated PBMCs was confirmed by a 6-h ^{51}Cr-release assay, and the representative results of the three patients (Pts.3, 6, and 9) are shown in Figure 5. These PBMCs showed significant levels of cytotoxicity against all the 11–18 cells (HLA-$A2^+$, $EGFR^+$) and SKOV3-A2 cells (HLA-$A2^+$, $EGFR^+$), but failed to kill any of the QG56 cells (HLA-A26, $EGFR^+$) or SKOV3 cells (HLA-A3/28, $EGFR^+$) tested. These PBMCs also failed to kill PHA-blastoid T cells (HLA-$A2^+$, $EGFR^-$). Peripheral blood mononuclear cells stimulated with an HIV peptide taken as a negative control did not show such HLA-A2-restricted cytotoxicity (data not shown). These results suggest that these PBMCs possess HLA-A2-restricted cytotoxicity reactive to $EGFR^+$ tumou cells.

Further, the restriction and peptide-specificity of the cytotoxicity were confirmed by inhibition and competition assays, respectively (Figure 6). Namely, levels of the cytotoxicity mediated by these peptide-stimulated PBMCs were significantly inhibited by anti-HLA-class I (W6/32) or anti-CD8 mAb, but not by the other mAbs tested in the assay. The cytotoxicity was also inhibited by the addition of the corresponding peptide-pulsed T2 cells, but not by addition of the HIV peptide-pulsed cells. These results suggest that the CTL activity is largely mediated by the peptide-specific $CD8^+$ T cells in an HLA-class I-restricted manner.

DISCUSSION

Among the 29 EGFR-derived peptides tested in this study, two peptides, one at position 479–488 and the other at position 1138–1147, were recognised by cellular and humoral immune responses in at least one-third of PBMCs and half of the sera samples from HLA-$A2^+$ epithelial cancer patients, respectively. These peptides, however, rarely induced CTL activity in the PBMCs of HDs, although IgG reactive to them was detectable in the sera of some HDs. The reactivity of PBMCs from several of HDs to EGFR peptides is not particularly surprising, given that EGFR is expressed not only in epithelial cancer cells but also in certain

Table 1 Humoral responses to the EGFR peptides

Subjects	HLA	Subtype	Responses to the EGFR peptides (OD values)[a]										
			$EGFR_{10-18}$	$EGFR_{61-70}$	$EGFR_{110-118}$	$EGFR_{479-488}$	$EGFR_{599-607}$	$EGFR_{653-662}$	$EGFR_{654-662}$	$EGFR_{729-738}$	$EGFR_{765-776}$	$EGFR_{852-861}$	$EGFR_{1138-1147}$
Pt.1	A2/24	A0207	—[b]	—	—	—	—	—	—	—	—	—	—
Pt.2	A2/24	A0206	—	—	—	—	—	—	—	—	—	—	—
Pt.3	A2/24	A0206	—	—	0.09	0.15	—	—	—	0.17	0.08	0.07	0.15
Pt.4	A2/11	A0206	0.26	—	0.08	0.49	—	—	—	0.10	—	—	0.10
Pt.5	A2	A0201	—	—	0.09	0.33	—	—	—	—	—	—	0.08
Pt.6	A2/24	A0206	—	—	—	0.22	—	—	—	0.24	0.10	—	0.14
Pt.7	A2/3	A0201	—	—	—	—	—	—	—	—	—	—	—
Pt.8	A2/24	A0206	—	—	—	0.07	—	—	—	—	0.16	—	0.07
Pt.9	A2/24	A0201	—	—	—	0.15	—	—	—	0.13	—	—	0.09
Pt.10	A2	A0207	—	0.07	0.14	0.12	0.13	0.07	—	0.07	—	—	0.13
Pt.11	A24/33		—	0.07	—	0.10	0.17	—	0.09	0.17	—	—	0.16
Pt.12	A24		—	—	—	0.21	0.08	—	—	—	—	—	0.07
Pt.13	A24		—	—	—	—	0.07	—	—	—	—	—	—
Pt.14	A24		—	—	—	—	—	—	—	—	0.18	—	
Pt.15	A24		—	—	—	0.55	—	—	—	0.59	—	—	—
Pt.16	A24		—	—	—	0.17	—	—	—	—	—	—	—
Pt.17	A24/31		—	—	—	0.24	—	—	—	0.10	—	0.07	0.10
Pt.18	A24		—	—	—	0.07	—	—	—	—	—	—	—
Pt.19	A24/33		—	—	—	—	—	1.22	—	0.31	—	—	—
Pt.20	A24/11		—	—	—	—	—	—	—	0.30	—	—	—
HD1	A2/24	A0206	—	—	—	—	0.20	—	0.07	—	—	—	0.13
HD2	A2/24	A0206	—	—	—	—	0.09	—	—	—	—	—	—
HD3	A2/11	A0206	—	—	—	0.26	—	—	—	—	—	—	—
HD4	A2/26	A0201	—	—	—	0.13	—	—	—	—	—	—	0.08
HD5	A2	A0206	—	—	—	—	—	—	—	—	—	—	—
HD6	A2/24	A0201	—	—	—	—	—	—	—	—	—	—	—
HD7	A24/33		—	—	—	—	—	—	—	—	—	—	—
HD8	A24/26		—	—	—	—	—	—	—	—	—	—	—
HD9	A24/26		0.07	0.07	—	—	0.20	—	0.12	—	—	—	0.26
HD10	A24		—	—	—	—	—	—	—	—	—	—	0.17
HD11	A11/33		—	0.09	0.09	—	0.13	—	0.07	—	—	—	0.15
Antipeptide Abs		Pt ($n=20$)	1	2	4	13	4	2	1	10	4	2	10
		HD($n=11$)	1	2	1	2	4	0	3	0	0	0	5

[a]Antipeptide IgG was assayed by ELISA as described in Materials and methods. Values represent the OD value at a serum dilution of 100 times. [b]The OD values lower than the cutoff (0.06) are shown as —.

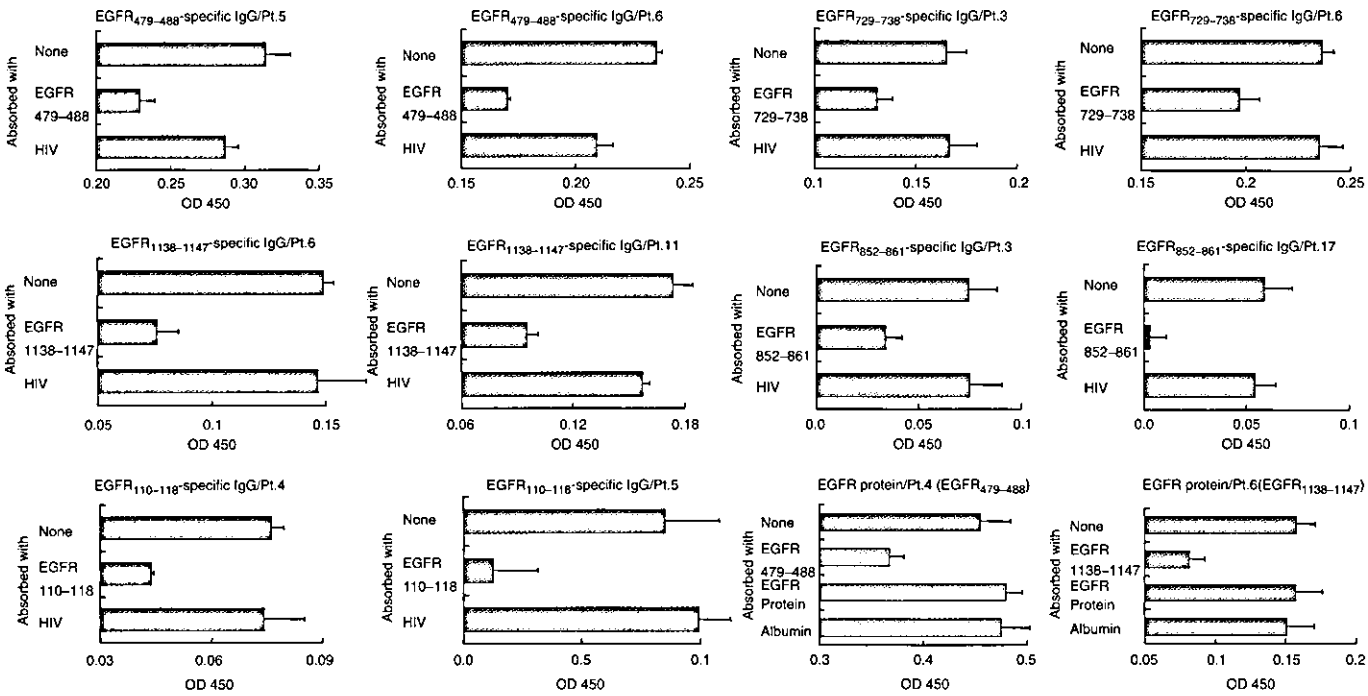

Figure 2 Specificity of antipeptide IgG. Each serum sample was absorbed with either a corresponding peptide or an HIV peptide used as a negative control three times at 37°C followed by testing of peptide-specific IgG activity with ELISA. Results of five peptides in the sera of each two representative patients from six (Pts. 3, 4, 5, 6, 11, and 17) are shown in the figure. In addition for testing of the peptide-specific IgG activity to whole protein, each serum sample was absorbed with an EGFR protein (purified from A431 cells), human albumin as a negative control, or a corresponding peptide as a positive control. The representative results from the sera of Pts.4 and 6 are shown in the lower, right-hand columns of this figure.

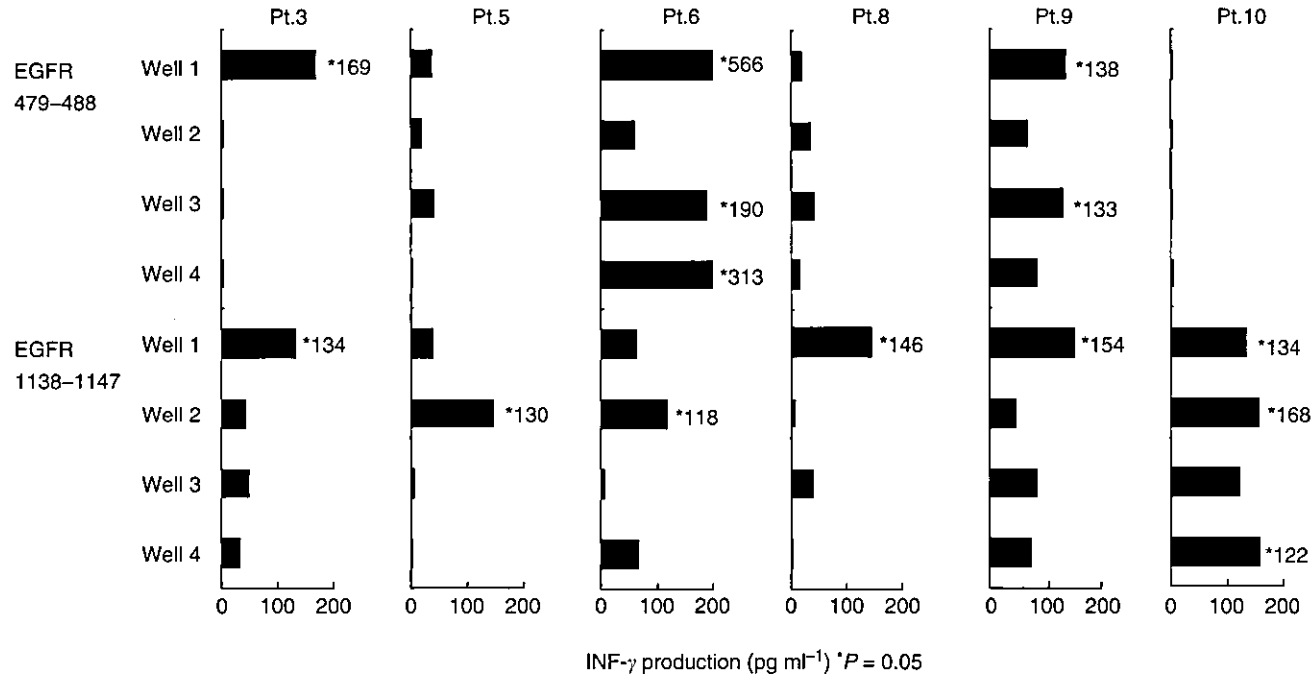

Figure 3 Cellular responses to peptide. Peptide-stimulated PBMCs from HLA-A2$^+$ cancer patients were cultured in four different wells (15×10^4 well^{-1}). On day 14 of culture, the peptide-stimulated PBMCs ($80-120 \times 10^4$ well^{-1}) from each well were independently collected and divided into four equal portions. Two such portions were separately tested for their ability to produce IFN-γ in response to T2 cells pulsed with a corresponding peptide, while the remaining two portions were tested with a negative control peptide (HIV). Background IFN-γ production in response to the HIV peptide (<50 pg ml^{-1}) was subtracted. An asterisk (*) indicates $P<0.05$ by a two-tailed Student's *t*-test. The representative results of six patients (Pts.3, 5, 6, 8, 9, and 10) are shown.

normal epithelial cells (Coussens *et al*, 1985; Yamamoto *et al*, 1986; Salomon *et al*, 1995). Humoral responses to EGFR in sera of patients with different malignancies were reported (Bei *et al*, 1999). Aberrant expression and activation of EGFR in malignant cells might lead to breakdown of immunotolerance. Cellular responses to HER2/neu-derived peptides are also detectable in PBMCs from

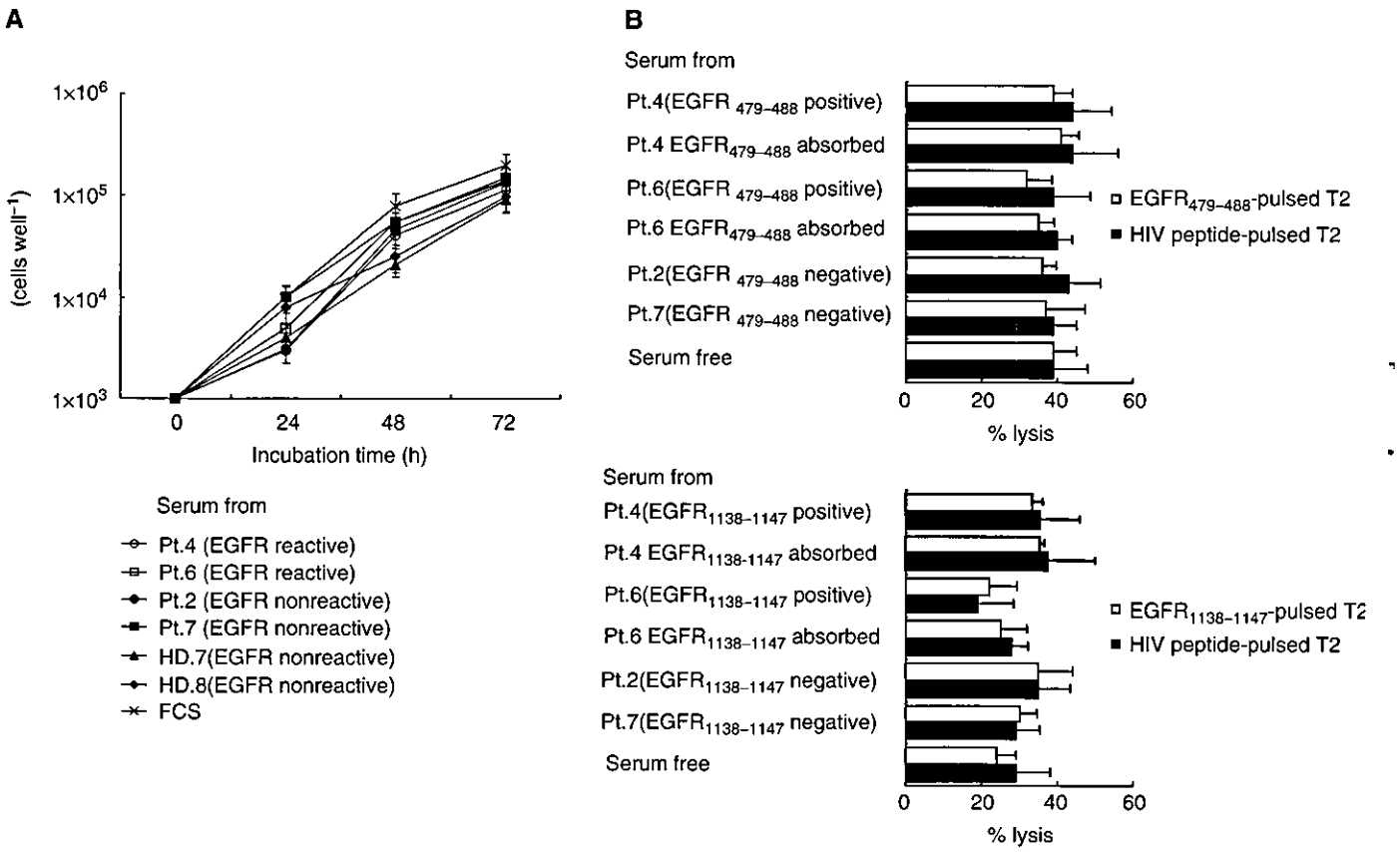

Figure 4 Direct inhibition and antibody-dependent cell-mediated cytotoxicity of sera possessing antipeptide IgG. (**A**) To test the direct growth inhibition activity of antipeptide IgG, 11–18 tumour cells were cultured in the presence of three different concentrations of sera, which had detectable levels of antipeptide activity. The 11–18 cells at 1×10^3 cells well^{-1} in a 96-well microculture plate (IWAKI, Chiba, Japan) were cultured for 12 h in the medium with 10% FCS followed by replacement of the culture medium to serum-free RPMI1640 with 1, 2, or 5% of serum possessing antipeptide activity. As controls, the same volumes of these sera absorbed with a corresponding peptide as well as sera possessing no antipeptide activity were used for the culture. The 11–18 cells were also cultured in the RPMI with 1, 2, and 5% FCS, respectively. After 24, 48, and 72 h incubation, the number of viable cells was determined by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The values are the mean ± s.e. of quadruplicate cultures. (**B**) To test the antibody-dependent cell-mediated cytotoxicity of antipeptide IgG, the cytotoxicity of freshly isolated PBMCs from HLA-A2$^+$ HDs against T2 cells pulsed with an EGFR-derived peptide or an HIV peptide as a negative control was measured in the presence of heat-inactivated serum possessing antipeptide activity by a standard 6-h ^{51}Cr-release assay. As controls, these sera absorbed with a corresponding peptide, sera possessing nonpeptide activity, and serum-free RPMI1640 medium were used. All sera used in the cytotoxicity assay were heat-inactivated at 56 C in 30 min. The standard 6-h ^{51}Cr-release assay was performed at three *E/T* (effector to target) ratios. The representative results on $EGFR_{479-488}$ and $EGFR_{1138-1147}$ peptides are shown in this figure (left side). The results were performed at *E/T* ratio 10 : 1, and the values represent the mean ± s.d. of % specific lysis in triplicate assays.

both cancer patients and HDs, whereas humoral responses to those HER2/neu peptides were not reported (Fisk *et al*, 1995; Peoples *et al*, 1995; Kawashima *et al*, 1999; Okugawa *et al*, 2000). It is of note, however, that at least one-third of PBMCs and sera samples from epithelial cancer patients in the present study showed both cellular and humoral responses to these two peptides, suggesting that these peptides have higher immunogenecity than any of the remaining 27 EGFR-derived peptides, which triggered immune responses only in a few subjects.

In addition to these two peptides, the four peptides, to which antipeptide IgGs were detectable in the sera of some cancer patients, were tested for their ability to induce peptide-reactive IFN-γ production in several cancer patients, but none of the four peptides induced the CTL activity under employed conditions. Among the four peptides, the $EGFR_{729-738}$ peptide was recognised by the majority of patients' sera, but by none of the sera of the HDs, suggesting that CD4$^+$ T cells of cancer patients may be involved in the antipeptide-specific IgG production. This point needs to be further studied in order to develop a monoclonal antibody to this epitope. Cellular responses to the remaining 23 peptides with HLA-A2 binding motifs were not investigated because of the limited number of PBMCs available for the analysis. Therefore, further studies will be needed to identify the EGFR-derived peptides capable of inducing HLA-A2-restricted cellular response alone.

We previously reported that IgG reactive against CTL epitope peptides was often detected in the prevaccination sera of cancer patients and also in the sera of HDs, and there was no obvious HLA-class I-A restriction involved (Ohkouchi *et al*, 2002; Kawamoto *et al*, 2003; Mine *et al*, 2003; Noguchi *et al*, 2003; Sato *et al*, 2003). Further, some CTL-directed peptides have shown the ability to elicit both cellular and humoral immune responses *in vivo* in phase I clinical studies, and levels of antipeptide IgG in postvaccination sera have well correlated with the overall survival of advanced cancer patients who received peptide vaccination (Mine *et al*, 2003; Sato *et al*, 2003). In contrast, IgG reactive to these CTL peptides has been reported to be either lacking or unbalanced in the sera of patients with atopic disease (Kawamoto *et al*, 2003). These results suggest that the IgG to these peptides play a role in host-defence against these diseases, although the underlying mechanism of the antitumour immune responses in cancer patients is presently unclear. The underlying mechanisms of IgG production against CTL epitope peptides in HDs as well as the disturbance of IgG production in patients with atopic disease

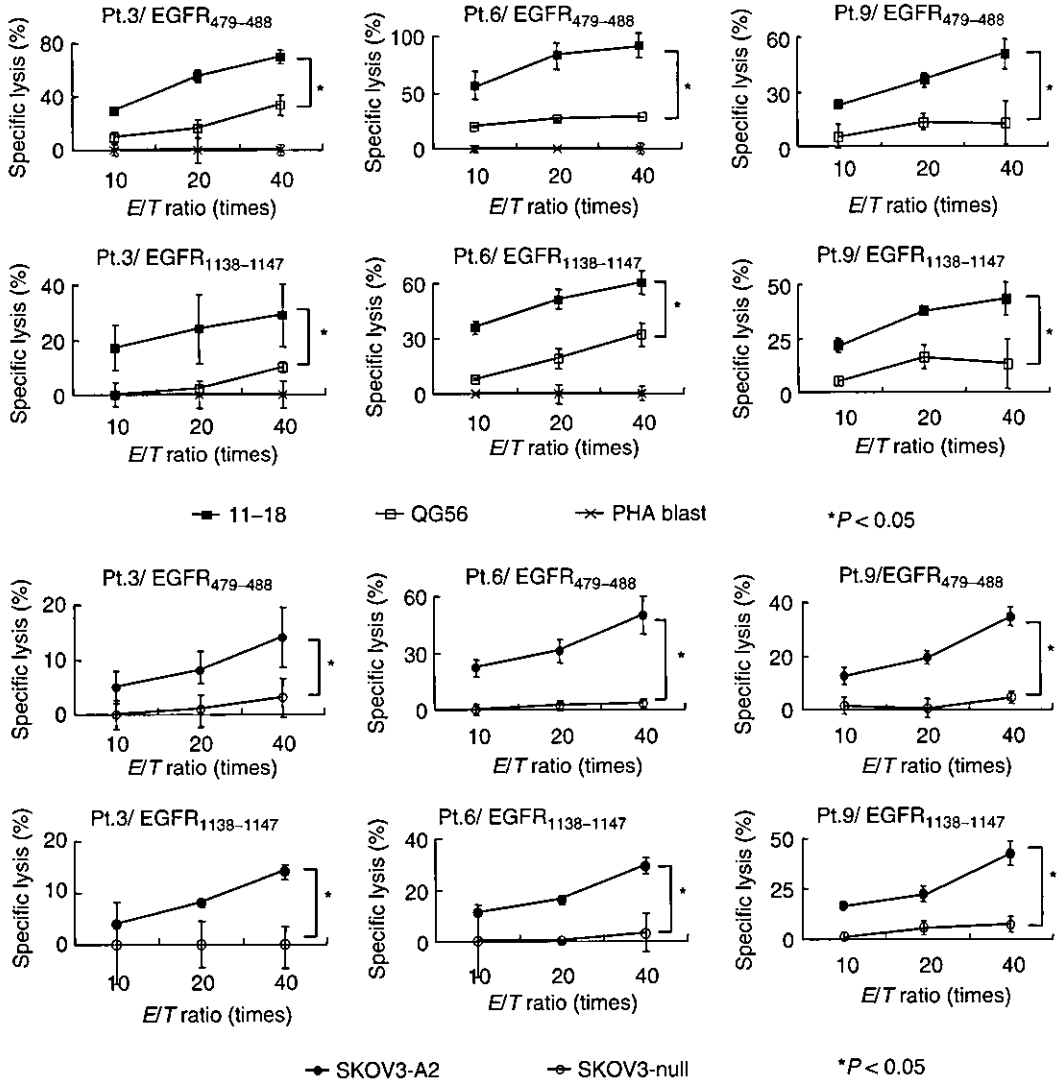

Figure 5 Cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against the following cancer cell lines: 11–18 (HLA-A2$^+$, EGFR$^+$), QG56 (HLA-A2$^-$, EGFR$^+$), SKOV3-A2 (HLA-A2$^+$, EGFR$^+$), and SKOV3 (HLA-A2$^-$, EGFR$^+$). PHA-blastoid T cells (HLA-A2$^+$, EGFR$^-$) were also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

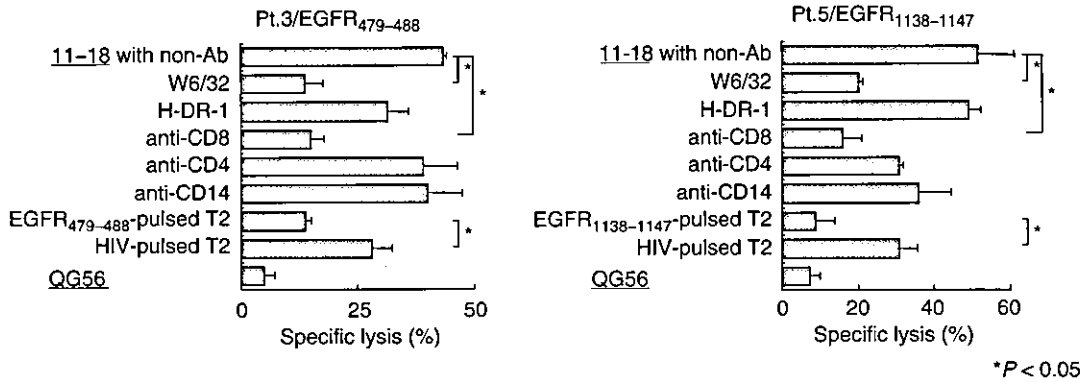

Figure 6 Inhibition and competition assays. Peptide-stimulated PBMCs were tested for their restriction and peptide-specificity of cytotoxicity against 11–18 (HLA-A2$^+$, EGFR$^+$) and QG56 (HLA-A2$^-$, EGFR$^+$) by the standard 6-h ^{51}Cr-release assay. In all, 20 μg/ml of anti-HLA-class I (W6/32, IgG2a),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

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

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

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

INTRODUCTION

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

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

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

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

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

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

PATIENTS AND METHODS

Patients

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

Patient-Oriented Peptide Vaccination

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

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

CombinationTherapy

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

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

Immunological Monitoring

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

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

Clinical Monitoring

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

Statistical Methods

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

RESULTS

Patient Characteristics

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

TABLE I. Patient Characteristics

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

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

Toxicity

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

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

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

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

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

Immunological Response During the CombinationTherapy

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

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

Clinical Response

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

TABLE III. Immune Response During the CombinationTherapy

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

(*Continued*)

TABLE III. (*Continued*)

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

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

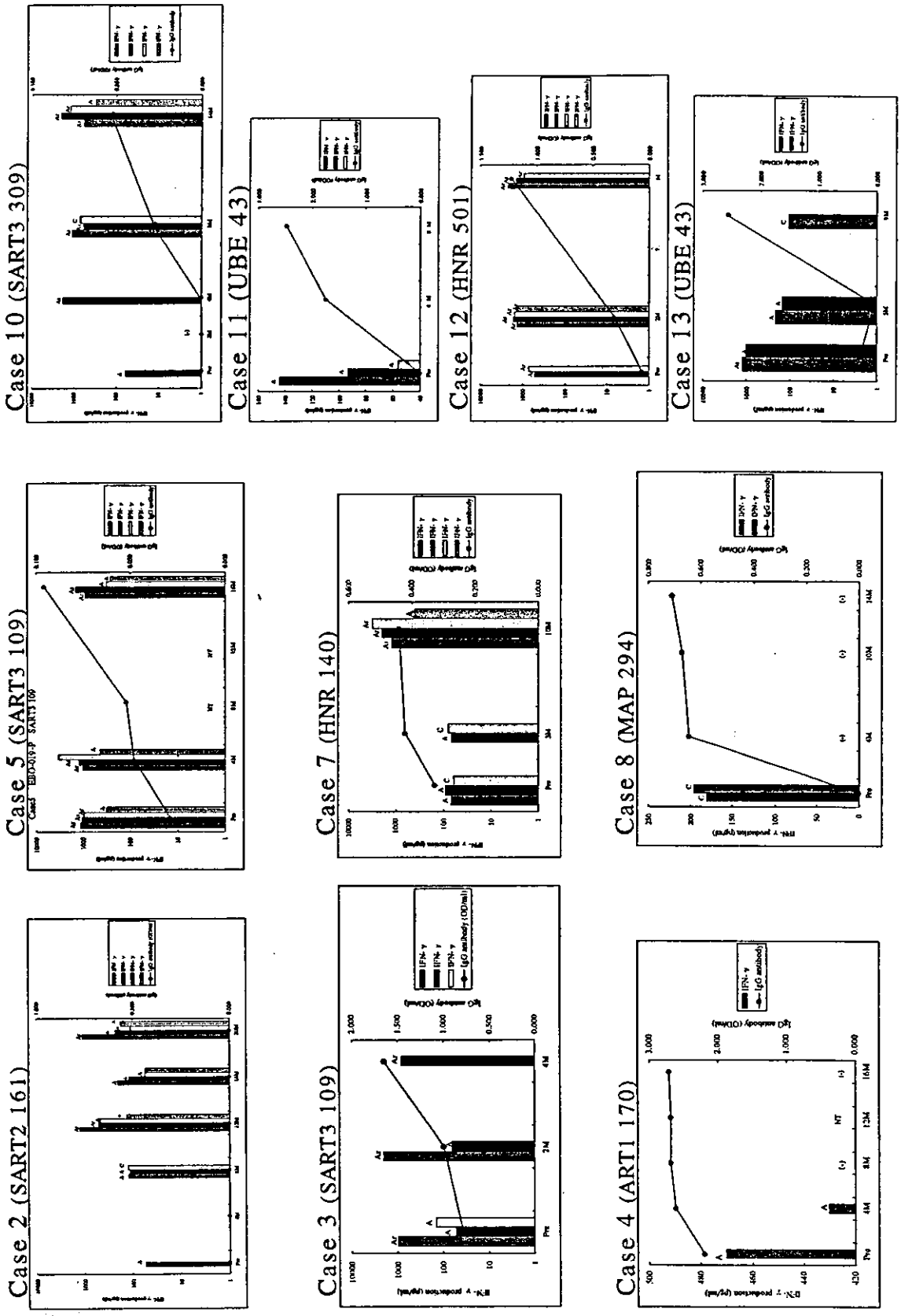

Fig. 1. Serial changes of IFN-γ productions and IgG levels specific for the peptides administered in each case. Augmentation of peptide-specific CTL precursors was observed in Cases 2, 5, 7, 8, 10, and 12, while induction of peptide-specific IgG was observed in Cases 3, 4, 5, 7, 8, 10, 11, 12, and 13.

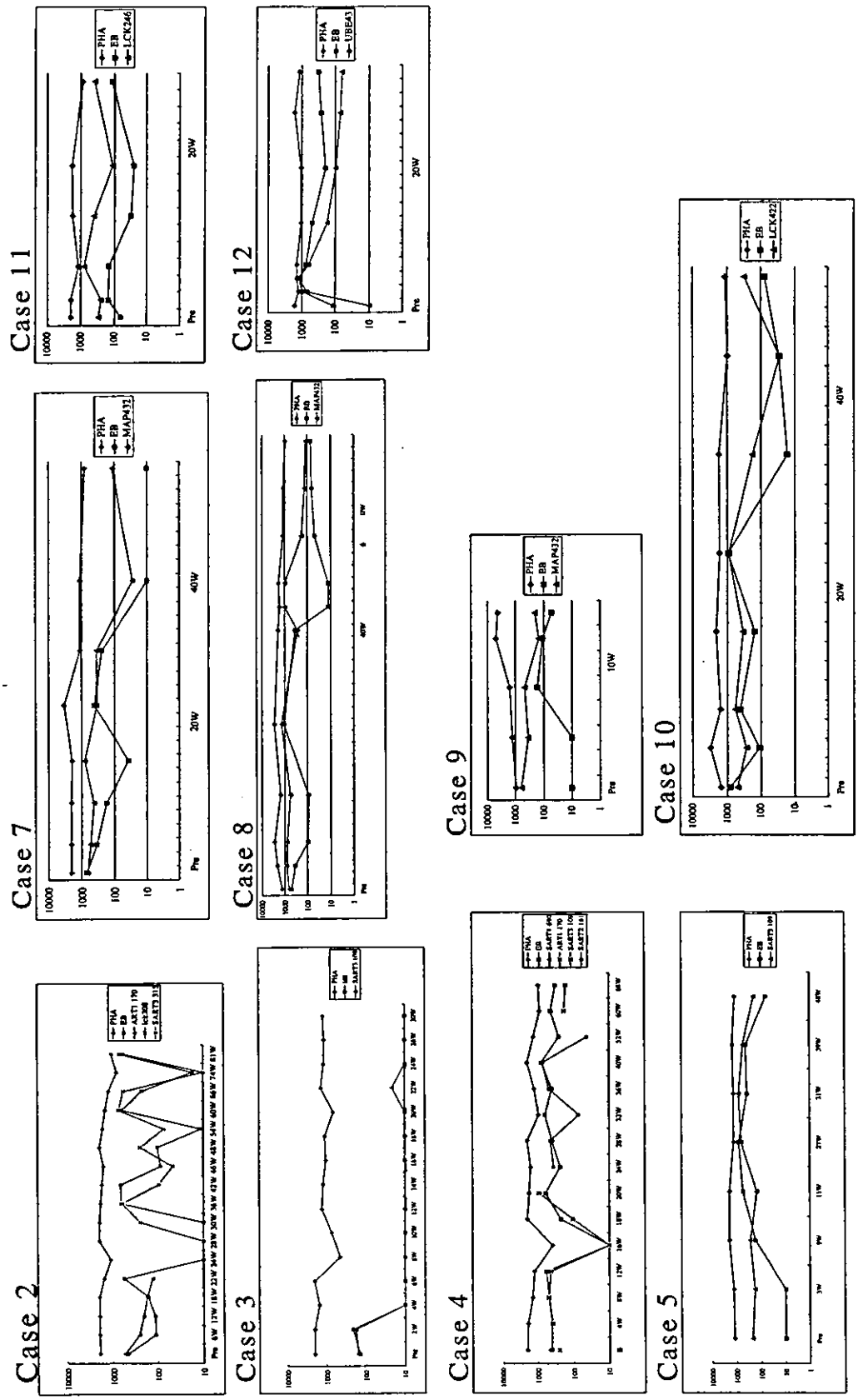

Fig. 2. Monitoring of the treatment-induced immune suppression. Cases 2 and 3 revealed severe immune suppression and their immune suppressions were recovered by discontinuing administration of full dose (560 mg/day) estramustine phosphate. There was no immune suppression in any of eight cases when the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