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Fig. 3A, B Cytotoxicity of $CD8^+$ T cells induced by in vitro stimulation with the PSCA peptides. A The PBMCs from 2 HLA-$A2^+$ healthy donors (*HD #4, HD #6*) and 2 HLA-$A2^+$ prostate cancer patients (*Pt #3, Pt #9*) were in vitro stimulated with the indicated PSCA peptides, as described in "Materials and methods." On day 15, half of the cultured cells were harvested, pooled from 8 wells, and cultured with T2 cells which were pulsed with the HIV peptide (*open symbols*) or the indicated PSCA peptide (*closed symbols*) for 18 h. The levels of IFN-γ in supernatants were then determined by ELISA. Thereafter, these cells were examined for their cytotoxicity against four kinds of tumor cell lines. Values represent the mean of triplicate assays. * $p < 0.05$ was considered statistically significant. B These PSCA peptide-stimulated CTLs from HD #6 and Pt #3 were examined for their cytotoxicity against PC93 and PC93-A2 cells with or without anti-class-I or anti-class-II mAb. Their cytotoxicity against PC93-A2 cells was also examined in the presence of unlabelled T2 cells, which were pre-pulsed with HIV or corresponding PSCA peptide. Values represent the mean of triplicate assays. * $p < 0.05$ was considered statistically significant

Cytotoxicity of PSCA peptide-stimulated $CD8^+$ T cells against prostate cancer cells

We further confirmed that PSCA peptide-specific $CD8^+$ T cells could show cytotoxicity against prostate cancer cells. As shown in Fig. 3A, PSCA peptide-stimulated PBMCs, which were derived from healthy donors 4 and 6 and prostate cancer patients 3 and 9, produced higher levels of IFN-γ in response to corresponding peptide-pulsed T2 cells; thereafter, $CD8^+$ T cells, which were positively purified from these PSCA-stimulated PBMCs, were examined for their cytotoxicity against four kinds of targets, including $PSCA^+$ HLA-$A2^-$ PC92, $PSCA^+$ HLA-$A2^+$ PC93-A2, $PSCA^-$ HLA-$A2^+$ colo201, and $PSCA^-$ HLA-$A2^-$ colo320. As a result, these $CD8^+$ T cells showed a higher level of cytotoxicity against PC93-A2 cells compared with the other three target cells. This observation was conformed by experiments using anti-class-I or anti-class-II mAb and by cold inhibition assay. As shown in Fig. 3B, cytotoxicity of purified $CD8^+$ T cells, which were stimulated with the corresponding PSCA peptide against PC93-A2 cells, were significantly inhibited by the addition of anti-class-I mAb, but not of anti-class-II mAb. In addition, their cytotoxicity against PC93-A2 cells were significantly suppressed when added with unlabelled corresponding PSCA peptide-pulsed T2 cells. These results indicate that the PSCA 7–15 and PSCA 21–30 peptides have the potential to generate HLA-A2-restricted $CD8^+$ T cells cytotoxic to prostate cancer cells.

IgG specific to the PSCA peptides

We previously reported that IgG reactive to CTL epitope peptides have been detected in patients with epithelial cancers and healthy donors [22, 23]. We therefore attempted to determine whether IgG reactive to PSCA-derived peptides can be detected in plasma of cancer patients and healthy donors (Table 3). IgG specific to the PSCA 21–30 peptide was detected in 9 of 12 cancer patients (75%). In patients 2, 5, and 10, IgG specific to the PSCA 43–51 or PSCA 70–79 peptide was detected. In patient 11, IgG reactive to the PSCA 43–51 or PSCA 108–117 peptide was detected. Representative results of cancer patients are shown in Fig. 3A. In healthy donors, IgG reactive to the PSCA 21–30 peptide was detected, but none of the other peptides were. Representative results for healthy donors are shown in Fig. 4B. The specificity of IgG against the PSCA 21–30 peptide was confirmed by an antibody absorption test. For example, it was found that the levels of anti-PSCA 21–30 peptide IgG in the plasma of patient 12 and healthy donor 1 were diminished by incubation with the PSCA 21–30 peptide-coated plate, but not with the HIV peptide-coated plate (Fig. 4C).

Recognition of glycine-substituted PSCA 21–30 peptide by IgG

Among 11 PSCA-derived peptides, both the PSCA 43–51 and PSCA 70–79 peptides contain one cysteine, and the PSCA 21–30 peptide has two cysteines. Cysteine can be easily oxidized by disulfide bondage, resulting in dimerization or cysteinylation [15, 18]; therefore, we attempted to exclude the possibility that preferential recognition of the PSCA 21–30 peptide by IgG is due to cysteine-based modification of the peptide (Fig. 5).

Table 3 Immunoglobulin G reactive to the PSCA peptides in plasma of HLA-$A2^+$ prostate cancer patients and healthy donors

Peptides	Prostate cancer patients													Healthy donor					
	1	2	3	4	5	6	7	8	9	10	11	12	Total	1	2	3	4	5	Total
PSCA4–13	–	–	–	–	–	–	–	–	–	–	–	–	0 of 12	–	–	–	–	–	0 of 5
PSCA5–13	–	–	–	–	–	–	–	–	–	–	–	–	0 of 12	–	–	–	–	–	0 of 5
PSCA7–15	–	–	–	–	–	–	–	–	–	–	–	–	0 of 12	–	–	–	–	–	0 of 5
PSCA21–30	+	+	+	–	+	+	+	–	+	+	–	+	9 of 12	+	+	+	+	+	5 of 5
PSCA43–51	–	+	–	–	+	–	–	–	–	+	+	–	4 of 12	–	–	–	–	–	0 of 5
PSCA70–79	–	+	–	–	+	–	–	–	–	+	–	–	3 of 12	–	–	–	–	–	0 of 5
PSCA106–115	–	–	–	–	–	–	–	–	–	–	–	–	0 of 12	–	–	–	–	–	0 of 5
PSCA108–117	–	–	–	–	–	–	–	–	–	–	+	–	1 of 12	–	–	–	–	–	0 of 5
PSCA109–117	–	–	–	–	–	–	–	–	–	–	–	–	0 of 12	–	–	–	–	–	0 of 5

Immunoglobulin G reactive to a corresponding peptide was judged to be positive when a difference of optical density at a 1:100 diluted plasma was more than 0.05

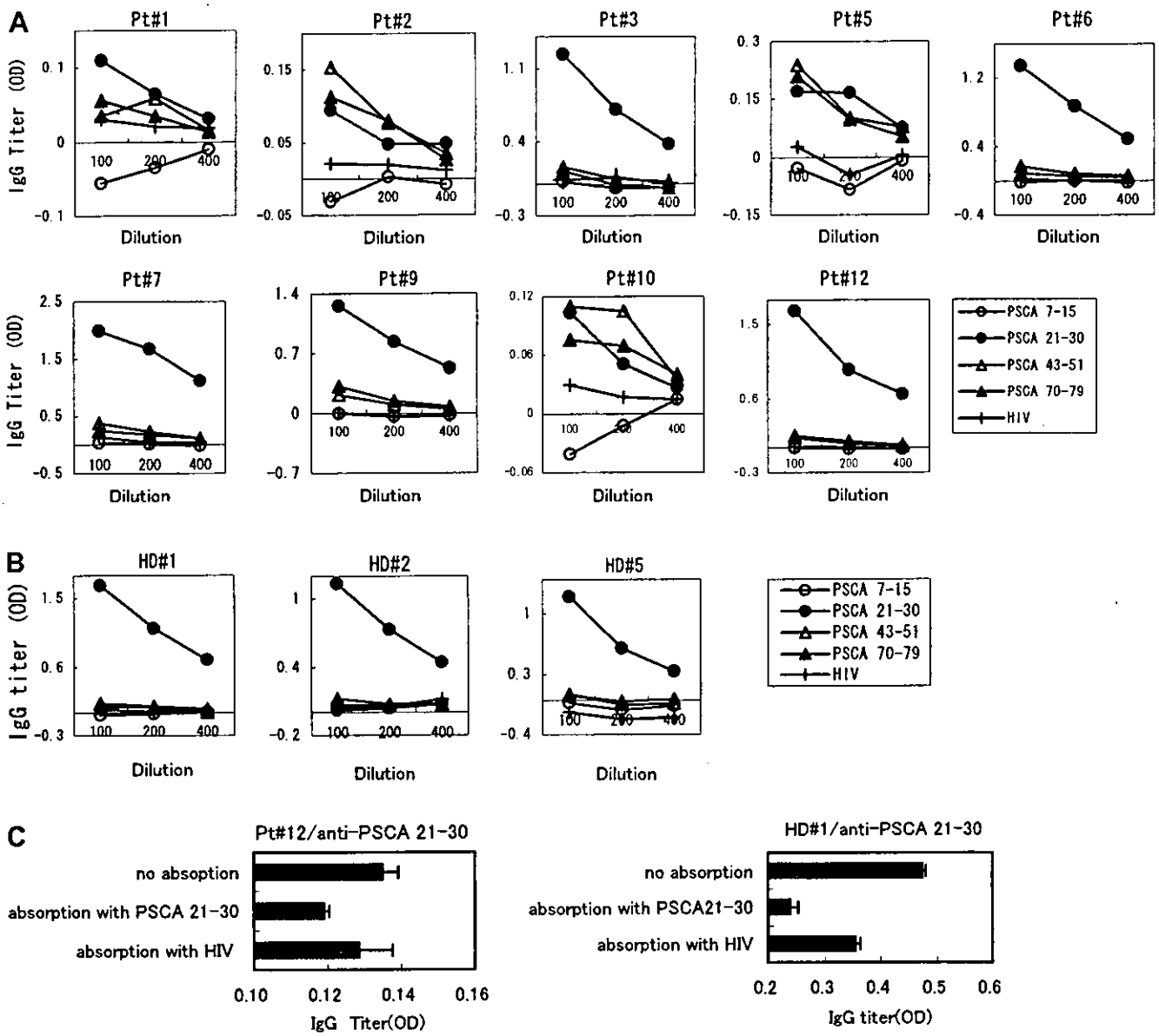

Fig. 4A–C IgG reactive to the PSCA peptides in plasma from prostate cancer patients and healthy donors. A The positive results of IgG reactive to PSCA peptides are shown. These values are shown as optical density (*OD*) units/ml, and the HIV peptide was used as a negative control. B Representative results of three healthy donors are shown. C To confirm the specificity of IgG to the PSCA 21–30 peptide, 100 µl of the sample plasma from patient 12 and healthy donor 1 were cultured in plates which were pre-coated with either the PSCA 21–30 peptide or the HIV peptide. Thereafter, the levels of IgG reactive to the PSCA 21–30 peptide in the resultant samples were determined by ELISA

Either or both cysteines at the third and sixth positions were replaced by glycine, and glycine-substituted peptides were found to be recognized by IgG of plasma from cancer patient 12 and healthy donor 4. Glycine-substitution did not inhibit recognition by IgG, but rather augmented recognition when the third position was substituted by glycine.

Discussion

Both the PSCA 14–22 and PSCA 105–113 peptides had been reported to be capable of inducing peptide-specific and prostate cancer-reactive CTLs from HLA-A2$^+$ patients [4, 14]. We first screened 11 peptide candidates, including these two peptides, but both the PSCA 14–22 and PSCA 105–113 peptides failed to induce peptide-specific CTLs from PBMCs of HLA-A2$^+$ cancer patients. On the contrary, our results identified both the PSCA 7–15 and PSCA 21–30 peptides as candidates for peptide-based immunotherapy for HLA-A2$^+$ prostate cancer patients. The PSCA 7–15 peptide has not been found to be immunogenic in studies from the other laboratories [4, 14], but the PSCA 21-30 peptide had not previously been tested for its ability to induce peptide-specific CTLs. One explanation for these different results may simply be due to the different culture

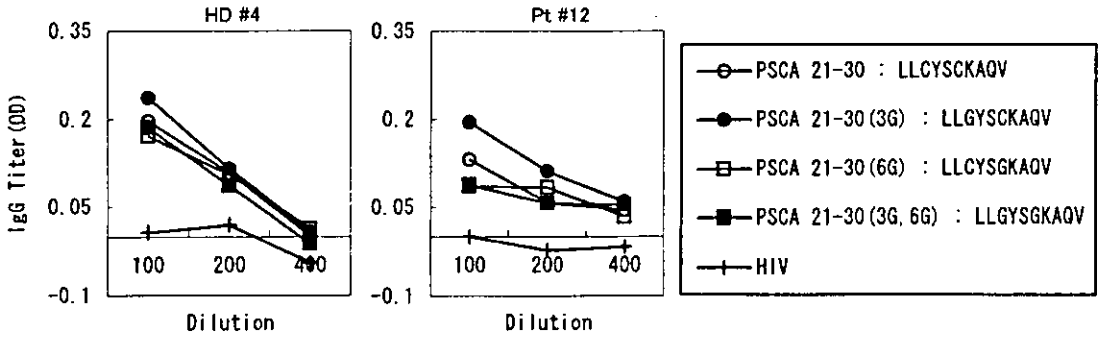

Fig. 5 Recognition of glycine-substituted PSCA 21–30 peptides by IgG. Either or both of the cysteines in the PSCA 21–30 peptide were substituted by glycine. It was then determined whether these modified PSCA 21–30 peptides could be recognized by IgG of plasma from healthy donor 4 and cancer patient 12. The levels of peptide-reactive IgG were determined by ELISA

protocols for in vitro CTL induction. Another possibility may be the HLA-A2 subtypes differing between Caucasians and Japanese. Most Caucasians are HLA-A*0201-positive, but HLA-A2 subtypes vary considerably in Japanese, as shown in Tables 1 and 2. The PSCA-derived peptides were prepared based on the binding motif to HLA-A*0201 molecules [24], and both the T2 cells and PC93-A2 cells express HLA-A*0201 molecules; however, the latter possibility is less likely since CTLs reactive to PSCA-derived peptides were induced not only from HLA-A*0201$^+$ patients, but also from those with other HLA-A2 subtypes, including HLA-A*0206 or HLA-A*0207 (Table 1). We have previously reported that epithelial tumor antigen-derived peptides, which were prepared based on the binding motif to HLA-A*0201 molecules, are also immunogenic in patients with several HLA-A2 subtypes [11, 13, 29]. These results suggest that both the PSCA 7–15 and PSCA 21–30 peptides are immunogenic in patients with HLA-A*0201, HLA-A*0206, and HLA-A*0207, which could cover most HLA-A2$^+$ patients throughout the world.

We tested 11 PSCA peptides and identified that the PSCA 7–15 and 21–30 peptides have the potential to induce prostate cancer-reactive CTLs. As is the case with other prostate-associated antigens [7, 8, 16], CTLs reactive to PSCA-derived peptides could be induced from HLA-A2$^+$ healthy donors. A similar phenomenon was observed with regard to melanocyte differentiation antigens [26]. The CTLs reactive to melanocyte differentiation antigens can be detectable in PBMCs of HLA-A2$^+$ healthy donors. These findings may suggest that CTLs reactive to non-mutated self antigens circulate in the peripheral blood of both certain healthy donors and cancer patients, and that active stimulation of such CTL precursors could result in effective immunotherapy. On the other hand, the PSCA 7–15 peptide was immunogenic in prostate cancer patients but not in healthy donors. It may be that CTL precursors reacting to the PSCA 7–15 peptide might be efficiently primed only in HRPC patients. This finding suggests that PSCA 7–15 is a promising molecule with regard to the induction of prostate cancer-reactive CTLs in HRPC patients.

The PSCA 21–30 peptide was recognized by both cellular and humoral immune responses in patients and healthy donors. In particular, IgG specific to this peptide was detected in 75% of cancer patients and in all five healthy donors. We have previously reported that IgG reactive to CTL epitope peptides is often detected in cancer patients and healthy donors [22, 23]. We also reported that IgG reactive to the PSA 248–257 peptide was frequently detectable in HLA-A24$^+$ healthy donors and prostate cancer patients [7]. The detection of IgG reactive to PSCA epitopes might reflect elicitation of immune responses toward the PSCA in both cancer patients and healthy donors.

It is important to determine whether plasma containing IgG reactive to the PSCA peptide could react positively to the LNCap cells and PC93 cells, and whether the LNCap cells and PC93 cells could absorb IgG reactive to the PSCA peptide. We tested these possibilities and found that no specific absorption of IgG reactive to the PSCA 21–30 peptide was observed when the plasma of reactive patients were incubated with these tumor cell lines, and that the heat-inactivated plasma containing IgG specific to the PSCA 21–30 peptide could not block cytotoxicity of PSCA 21–30 peptide-induced CTLs against the PC93-A2 cells (unpublished observation). Although we have no clear understanding of the roles peptide-specific IgG play in anti-tumor immune responses in cancer patients, it may be interesting to clarify the actions of IgG against CTL-peptides in cancer patients.

We examined the surface expression of PSCA on the LNCap, PC93, and two colon carcinoma cell lines. The PC93 was positive, but the LNCap was concluded to be negative for the surface expression of the PSCA, although a part of LNCap was non-specifically stained with anti-PSCA mAb. This result is consistent with a previous report that the LNCap cells are not to express PSCA on their cell surface [6]; however, our result of RT-PCR revealed that the LNCap cells expressed the mRNA expression of PSCA. Actually, we isolated a cDNA encoding PSCA gene by the PCR method from cDNA of the LNCap cells (unpublished observation). The LNCap cells might express the PSCA in their cytoplasm.

Conclusion

In conclusion, we identified new two PSCA-derived peptides immunogenic in HLA-A2$^+$ prostate cancer patients. This information could extend the possibility of treating HLA-A2$^+$ HRPC patients using peptide-based specific immunotherapy.

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A phase I trial of cytotoxic T-lymphocyte precursor-oriented peptide vaccines for colorectal carcinoma patients

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In most protocols of peptide-based vaccination, no consideration has been paid to whether or not peptide-specific cytotoxic T-lymphocyte (CTL) precursors are pre-existent in cancer patients. Initiation of immune boosting through vaccination is better than that of immune priming to induce prompt and strong immunity. In this study, 10 human histocompatibility leukocyte antigen-A24$^+$ patients with advanced colorectal carcinomas were treated with up to four peptides that had been positive for pre-vaccination measurement of peptide-specific CTL precursors in the circulation (CTL precursor-oriented peptide vaccine). No severe adverse effect was observed, although local pain and fever of grade I or II were observed. Post-vaccination peripheral blood mononuclear cells (PBMCs) from five patients demonstrated an increased peptide-specific immune response to the peptides. Increased CTL response to cancer cells was detected in post-vaccination PBMCs of five patients. Antipeptide immunoglobulin G became detectable in post-vaccination sera of seven patients. Three patients developed a positive delayed-type hypersensitivity response to at least one of the peptides administrated. One patient was found to have a partial response; another had a stable disease, sustained through 6 months. These results encourage further development of CTL precursor-oriented vaccine for colorectal cancer patients.
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Recent advances in molecular biology and cellular immunology have resulted in identification of various antigens and epitopes recognised by human histocompatibility leukocyte antigen (HLA)-class-I-restricted cytotoxic T lymphocytes (CTLs) from various cancers (Bruggen *et al*, 1991; Brichard *et al*, 1993; Kawakami *et al*, 1994a, b Fisk *et al*, 1995; Peoples *et al*, 1995; Robbins *et al*, 1996; Correale *et al*, 1997; Correale *et al*, 1998). Many clinical trials of peptide-based immunotherapy have shown an increased immune response to the vaccinated peptides (Rosenberg *et al*, 1998; Marchand *et al*, 1999; Jager *et al*, 2000; Gajewski *et al*, 2001; Gjertsen *et al*, 2001; Lau *et al*, 2001; Valmori *et al*, 2001). However, these initial clinical studies have produced few clinical responses in the various types of cancer treated including melanoma and colorectal cancer (Finn and Lotze, 2001; Miyagi *et al*, 2001; Sadanaga *et al*, 2001). This failure could be in part due to the insufficient and late stages of CTL induction by the current regimen, in which pre-vaccination screening of suitable peptides for each patient among a large number of candidate peptides is not conducted. As a result, vaccination in the present study began with immune priming. This standard regimen could be effective in preventing infectious disease. However, the main goal of a cancer vaccine is treatment of malignant disease. The time-lag necessary for priming of an antitumour response should be seriously considered, as the expected survival of most advanced cancer patients under these regimens is 3–9 months (Cole and Rodu, 2001; Miyagi *et al*, 2001). Therefore, the development of a new regimen of therapeutic vaccine is needed (Finn and Lotze, 2001). One regimen might include pre-vaccination measurement of peptide-specific CTL precursors in the circulation, followed by vaccination of only CTL-reactive peptides (CTL precursor-oriented vaccine). We have previously reported 14 vaccine candidate peptides that can induce HLA-A24-restriced and tumour-specific CTL in cancer patients (Gomi *et al*, 1999; Kikuchi *et al*, 1999; Yang *et al*, 1999; Harashima *et al*, 2000; Kawano *et al*, 2000; Nakao *et al*, 2000; Nishizaka *et al*, 2000). We have also shown that most cancer patients have peptide-specific CTL precursors for some of these peptides, and that peripheral blood mononuclear cells (PBMCs) stimulated with positive peptides show HLA-class-I-restricted and tumour-specific cytotoxicity (Hida *et al*, 2002; Maeda *et al*, 2002; Suzuki *et al*, 2002). In the present study, patients with advanced stages of colorectal cancer were immunised with up to four peptides identified in pre-vaccination measurement of peptide-specific CTL precursors to evaluate the toxicities and responses to CTL precursor-oriented peptide vaccination.

MATERIALS AND METHODS

Patients and eligibility criteria

The study protocol was approved by the Institutional Ethical Review Boards of Hokkaido University and Kurume University, respectively. Complete written informed consent was obtained

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from all of patients at the time of enrolment. According to the protocol, patients were required to be HLA-A24 positive, and to have a histologically confirmed lesion of colorectal carcinoma. Eligibility criteria included an age of 85 years or less, serum creatinine of less than 1.4 mg dl^{-1}, bilirubin of less than 1.5 mg dl^{-1}, a platelet count of 100 000 μl^{-1} or more, haemoglobin of 8.0 g dl^{-1} or more, and total WBC of 3000 μl^{-1} or more. Hepatitis B surface antigen, Hepatitis C antigen, and human immunodeficiency virus (HIV) were required to be negative. The patients, who had been untreated for at least 4 weeks before the study, had an Eastern Cooperative Oncology Group performance status of 0–2. The treatment was carried out at the First Department of Surgery, Hokkaido University School of Medicine (patients 1–9) and the Department of Surgery, Kurume University School of Medicine (patient 10) from November 2000 through April 2002. All immunological analyses were carried out at the Department of Immunology, Kurume University School of Medicine.

Screening of peptide-specific CTL precursors

A volume of 30 ml of peripheral blood was obtained pre- and post- (3rd, 6th, and 9th) vaccination, and PBMCs were isolated by means of Ficoll–Conray density gradient centrifugation, as reported previously (Miyagi *et al*, 2001). A previously reported method was used to detect peptide-specific CTL precursors in PBMCs (Hida *et al*, 2002; Suzuki *et al*, 2002). Briefly, PBMCs (1×10^5 cells $well^{-1}$) were incubated with 10 μM of a peptide in wells of 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 (GIBCO BRL), 10% FCS, 100 U ml^{-1} of interleukin (IL)-2, and 0.1 μM MEM nonessential amino-acid solution (GIBCO BRL). Every 3 days, half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM). The assay was performed in quadruplicate. After a 12-day incubation, the cultured cells in one well were divided into four wells, two of which were used for corresponding peptide-pulsed C1R-A2402 cells, and the other two of which were used for HIV peptide (RYLRQQLLGI)-pulsed C1R-A2402 cells. The HLA-A24-binding HIV peptide was used as a negative control. After an 18-h incubation period, the supernatants were collected and the level of interferon (IFN)-γ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg ml^{-1}). The background response to HIV peptide-pulsed C1R-A2402 cells was subtracted from the value. Assessment of peptide-specific CTL precursors was performed based on two parameters, the *P*-value by the Student's *t*-test and IFN-γ production, as described in legends of Tables 2 and 4. According to the results of this test, up to four positive peptides were selected for each patient, utilised in a skin test, and then, if negative, injected as a vaccination. The screening of peptide-specific CTL precursors was performed by the same method after the 6th vaccination to evaluate the effects of immunisation.

Peptides and vaccination

The peptides utilised in the present study were prepared under conditions of Good Manufacturing Practice using a Multiple Peptide System (San Diego, CA, USA). Montanide ISA-51, an incomplete adjuvant, was manufactured by Seppic, Inc (Franklin Lakes, NJ, USA). The peptides were supplied in vials containing 3 mg ml^{-1} sterile solution for injection. A 3 mg portion of peptide with sterile saline was added in a 1 : 1 volume to Montanide ISA-51, then mixed in a Vortex mixer (Fisher, Inc, Alameda, CA, USA). The resulting emulsion was injected subcutaneously into the lateral thigh using a glass syringe. Patients were vaccinated every 14 days for a total of three injections to measure the toxicity. For the patients with no toxicity, the vaccinations were repeated biweekly up to 15 times with informed consent from each patient.

Delayed-type hypersensitivity (DTH) skin test

Skin tests were performed using 50 μg of each peptide injected intradermally in a volume of 100 μl using a tuberculin syringe and a 27-gauge needle. Saline was a negative control for assessment of DTH. At least 7 mm of induration or erythema read 48 h after injection was needed to score the skin test as positive (Nestle *et al*, 1998).

Assay of cytotoxicity

Cytotoxic activity was measured using a standard 6-h ^{51}Cr-release assay (Gomi *et al*, 1999; Miyagi *et al*, 2001). Cryopreserved PBMCs were thawed and cultured in the medium consisting of 45% RPMI-1640 medium, 45% AIM-V® medium (GIBCO BRL), 10% FCS, 100 U ml^{-1} of IL-2, and 0.1 μM MEM nonessential amino-acid solution (GIBCO BRL). On the 14th day of culture, the cells were harvested and served for the assay. To avoid the bias of bioassays, PBMCs harvested at different times from a single patient were thawed at the same time. SW620 (HLA-A24$^+$, colon adenocarcinoma), COLO201 (HLA-A24$^-$, colon adenocarcinoma), and PHA-blastoid T cells (HLA-A24$^+$) were used as target cells (1×10^3 $well^{-1}$), and 50-fold unlabelled K562 cells were added into wells to decrease nonspecific killing activity.

Purification of CD8$^+$ or CD4$^+$ T cells

CD8$^+$ or CD4$^+$ T cells were positively isolated from peptide-stimulated PBMCs using the CD8 Positive Isolation Kit (DYNAL, Oslo, Norway) or the CD4 Positive Isolation Kit (DYNAL) according to the manufacturer's instructions. In both cases, the percentage of purified CD8$^+$ or CD4$^+$ T cells was more than 90% (data not shown).

Kinetics of peptide-specific CTL precursors

To detect the kinetics of peptide-specific CTL precursor frequency in patient 1, PBMCs from before and after vaccination were incubated at 100 cells per well of a 96-well u-bottom microculture plate in the presence of feeder cells without the peptides. Cells from each well were harvested on the 14th day of culture and tested for their ability to produce IFN-γ by recognition of peptide-pulsed CIR-A2402 cells in duplicate assay. The well was considered positive if it contained effector cells producing much higher than 100 pg ml^{-1} and also statistically significant levels ($P < 0.05$ by the Student's *t*-test) of IFN-γ in response to CIR-A2402 cells preloaded with a corresponding peptide as compared with those in response to the HIV peptide-pulsed CIR-A2402 cells.

Detection of serum immunoglobulin G (IgG) levels

An ELISA was used to detect the serum IgG levels specific to the peptides administered, as reported previously (Miyagi *et al*, 2001). Briefly, the peptide (20 μg $well^{-1}$)-immobilised plate was blocked with Block Ace (Yukijirushi, Tokyo, Japan), washed, and 100 μl $well^{-1}$ of serum samples diluted with 0.05% Tween 20-Block Ace were added to the plate. After a 2-h incubation, the plate was washed and further incubated for 2 h with an 1 : 1000-diluted rabbit anti-human IgG (DAKO, Glostrup, Denmark). The plate was washed, after which 100 μl of 1 : 100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase-dextran polymer (En Vision, DAKO) was added to each well, and the plate was incubated for 40 min. After washing, 100 μl $well^{-1}$ of tetramethyl-benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of 1 M phosphoric acid. To estimate peptide-specific IgG levels, the optical density values of each sample were compared with those of serially diluted standard samples, and the values are shown as the optical density. The

specificity of the ELISA was tested as follows. Serum samples (1:100 diluted) were incubated in wells precoated with corresponding or irreverent peptides to absorb the peptide-specific IgG. After a 2-h incubation at room temperature, samples were transferred to new wells precoated with the sample peptide used in the first absorption, and this protocol was repeated for a total of three times. The samples were then subjected to peptide-specific IgG ELISA.

Evaluation of treatment response

All known sites of disease were evaluated by CT-scan or X-ray examination pre- and post-vaccinations (the 3rd, 6th, 9th, and 12th). Patients were assigned a response category according to the response evaluation criteria in solid tumours, a revised version of the WHO criteria published in the WHO Handbook for reporting results of cancer treatment in June 1999.

RESULTS

Demographics of the patients

In all, 10 patients with advanced colorectal carcinomas were enroled in this phase I study. Demographic details of the patients are shown in Table 1. The median patient age was 67 years (range 28–78). All patients had liver, lung, peritoneum, or lymph node metastases. All patients underwent surgical resection of the primary lesion; nine had failed previous chemotherapy. All 10 patients completed the first three vaccinations within the protocol under informed consent, and all of them received more vaccinations (6–15) under additional informed consent.

Screening of peptide-specific CTL precursors

All tumour-related antigens were identified by screening of a cDNA library from tumour cells using tumour-reactive CTLs. The peptides used for this study have the potential to induce HLA-A24-restricted and tumour-specific CTL activity in PBMCs of HLA-A24$^+$ cancer patients (Kikuchi *et al*, 1999; Yang *et al*, 1999; Gomi *et al*, 1999; Harashima *et al*, 2000; Kawano *et al*, 2000; Nakao *et al*, 2000; Nishizaka *et al*, 2000). Pre-vaccination PBMCs were provided for screening of the CTL precursors reactive to the 14 candidate peptides, followed by selection of peptides based on evaluation of the results with the criteria shown in Table 2. The assay was performed in quadruplicate. After a 12-day incubation, the cultured cells in one well were separated into four wells, two of which were used for corresponding peptide-pulsed C1R-A2402 cells, and the other two of which were used for HIV peptide-pulsed C1R-A2402 cells. The HLA-A24-binding HIV peptide was used as a negative control. The assessment of peptide-specific CTL

Table 1 Patient characteristics

Patient	Age	Sex	Primary site	Metastasis site	Previous treatment[a]	P.S.[b]	No. of vaccination received[c]
1	67	M	Transverse colon	Liver, para-aortic lymph node	S. C	0	15
2	38	F	Transverse colon	Liver, peritoneum (intrapelvic)	S. C	0	12
3	67	M	Ascending colon	Lung, liver	S. C	1	6
4	78	M	Rectum	Liver	S. C. M	0	10
5	70	F	Sigmoid colon	Lung, liver	S. C	1	11
6	72	M	Rectum	Liver	S	1	10
7	67	M	Sigmoid colon	Lung	S. C	0	8
8	44	M	Sigmoid colon	Lung, liver	S. C	0	6
9	46	M	Ascending colon	Peritoneum (intrapelvic)	S. C	0	6
10	28	F	Sigmoid colon	Peritoneum (intrapelvic)	S. C	1	8

[a]S = surgery; C = chemotherapy; M = percutaneous microwave coagulation therapy. [b]P.S. = performance status by ECOG score. [c]The initial protocol consisted of three vaccinations; additional vaccinations were subsequently performed to patients who showed a favourable clinical course after they provided further informed consent.

Table 2 Pre-vaccination screening of peptide-specific CTL precursors

		Patients											
Peptide	Sequence	1	2	3	4	5	6	7	8	9	10	Positive case	Vaccinated case
SART1 690	EYRGFTQDF								OA	OAA		2/10	2/10
SART2 93	DYSARWNEI								OAB			1/10	1/10
SART2 161	AYDFLYNYL								OA	OAA		2/10	2/10
SART2 899	SYTRLFLIL	●B										1/10	0/10
SART3 109	VYDYNCHVDL	OA	OB			OC	OC	OA		OA		6/10	6/10
SART3 315	AYIDFEMKI							OC			OC	2/10	2/10
CyB 84	KFHRVIKDF							●A				1/10	0/10
CyB 91	DFMIQGGDF			OA	OA			OC			OA	4/10	4/10
lck 208	HYTNASDGL	OC	OA		OA	OC		OC	OB		OAAA	7/10	7/10
lck 488	TFDYLRSVL		OAB		OAAAA					OAB	OA	4/10	4/10
lck 488	DYLRSVLEDF	OA		OA	OAA							3/10	3/10
ART1 170	EYCLKFTKL	●B			●A		●A			●AA		4/10	0/10
ART4 13	AFLRHAAL					●C						1/10	0/10
ART4 75	DYPSLSATDI			OA			OB					2/10	2/10

White circles indicate that the peptide was positive for the CTL precursor induction assay and was injected. Black circles indicate that the peptide was positive for the CTL precursor induction assay but was not administrated due to immediate-type hypersensitivity by skin test. The 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: A, $P<0.05$ and IFN-γ production $>50\,pg\,ml^{-1}$; B, $P<0.05$ and $50\,pg\,ml^{-1}$ $>$ IFN-γ production $>25\,pg\,ml^{-1}$; C, $0.05<P<0.1$ and IFN-γ production $>25\,pg\,ml^{-1}$. The classification is shown by letters of the alphabet, and each character represents the results of each well. For example, ABC means that three wells were judged as A, B, and C, and one well was negative.

precursors was performed based on two parameters, the *P*-value by the Student's *t*-test and IFN-γ production, as shown in the table legend. When these peptides were found to induce immediate-type hypersensitivity by a skin test, a fifth peptide was vaccinated if it proved negative in the skin test. $SART2_{899}$, CyB_{91}, $ART1_{170}$, and $ART4_{13}$ were positive for immediate-type hypersensitivity in all patients tested and were not injected at all. As a result, five patients were injected with four peptides, three patients with three peptides, and two with two peptides. The vaccinated peptides for each patient are shown in Table 2. It is noteworthy that the profiles of the vaccinated peptides varied greatly among the 10 patients.

Toxicities

All 10 patients were evaluated for toxicity; the overall toxicities are shown in Table 3. The vaccinations were generally well-tolerated, but almost all patients (eight out of 10) had grade I or II local redness and swelling at the injection sites. Fever with mild flu-like symptoms was observed in four patients (grade I or grade II), although this symptom was transient and no medication was needed. Grade I fatigue or nausea was observed in two patients, and grade I anorexia, diarrhoea, or vomiting was observed in one. No vaccine-related grade III or IV toxicity was observed (data not shown). There was no clinical evidence of an autoimmune reaction as determined by symptoms, physical examination, or laboratory test.

Cellular immune responses

Post-vaccination (6th) PBMCs showed increased amounts of peptide-specific IFN-γ production compared to pre-vaccination PBMCs in five out of 10 patients (1, 2, 5, 6, and 10), as described in Table 4. Representative results of patients 1 and 2 are shown in Figure 1A. In patients 1 and 2, CTL response to the lck_{208} was apparently induced after the 6th vaccination. In five other patients, peptide-specific CTL response decreased. We further tested the reactivity of purified $CD4^+$ or $CD8^+$ T cells in response to the administered peptides. The pre- or post-6th vaccination PBMCs from patient 2 were *in vitro* stimulated, and purified $CD4^+$ or $CD8^+$ T cells were tested for their reactivity to the $SART3_{109}$ peptide-pulsed C1R-A2402 cells. As shown in Figure 1B, purified $CD8^+$ T cells from the post-vaccination PBMCs of patient 2 produced IFN-γ in an antigen-specific manner, although no definite IFN-γ production specific to the $SART3_{109}$ peptide was observed when unseparated post-6th PBMCs from patient 2 were used (Figure 1A). Purified $CD4^+$ T cells failed to produce IFN-γ in a peptide-specific manner. On the other hand, no peptide-specific IL-4 production was observed in the case with purified $CD8^+$ or $CD4^+$ T cells (data not shown).

We next examined cytotoxicity of pre- and post- (3rd, 6th, and 9th) vaccination PBMCs from eight patients against SW620 (HLA-$A24^+$ colon tumour cells), COLO201 cells (HLA-$A24^-$ colon tumour cells), and PHA-activated T cells (HLA-$A24^+$) (Figure 2). Tumour-related antigens from which all peptides used in this study were derived are nonmutated self-antigens overexpressed in tumour cells, including SW620 and COLO201 (Shichijo *et al*, 1998; Yang *et al*, 1999; Gomi *et al*, 1999; Harashima *et al*, 2000; Kawano

Table 3 Toxicities associated with the peptide vaccination

Toxicities	Grade[a] I	Grade[a] II	Total
Anorexia	1		1
Dermatologic	7	1	8
Diarrhoea	1		1
Fatigue	2		2
Fever	2	2	4
Nausea	2		2
Vomiting	1		1

[a]Toxicities are based on the NIH Common Toxicity Criteria.

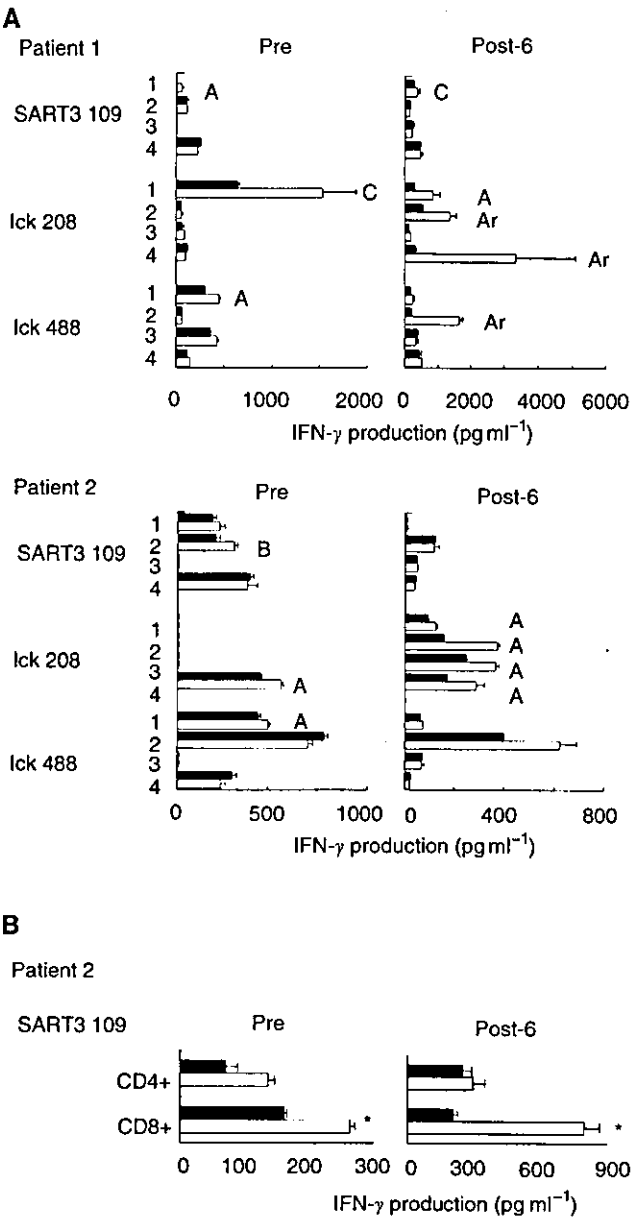


Figure 1 Assay of peptide-specific CTL precursors. (**A**) Pre- and post-(6th) vaccination PBMCs were provided for screening of reactivity to each of the 14 peptides listed in Table 2 in the quadruplicate assays. Representative results of patients 1 and 2 are shown in this figure. The peptide-stimulated PBMCs were cultured with C1R-A2402 cells that were preloaded with the corresponding peptide (open bar) or the HIV peptide (closed bar). The level of IFN-γ in the supernatant was determined by ELISA. The result was evaluated by the classification shown in the legend of Table 4. Each alphabet character represents the result of each well. (**B**) Pre- and post- (6th) vaccination PBMCs from patient 2 were stimulated *in vitro* with the $SART3_{109}$ peptide. The peptide-stimulated PBMCs were harvested, and positively isolated $CD4^+$ or $CD8^+$ T cells were cultured in triplicate with C1R-A2402 cells that were preloaded with the $SART3_{109}$ peptide (open bar) or the HIV peptide (closed bar). The level of IFN-γ in the supernatant was determined by ELISA. *Statistically significant at $P<0.05$.

Table 4 Summary of response to the peptide vaccination

Patient	Peptide	Peptide-specific CTL[a] Pre	Peptide-specific CTL[a] Post	Ab to peptide Pre	Ab to peptide Post[b]	DTH Pre	DTH Post[b]	Clinical response/ Time to progression (months)
1	SART3 109	A	C	–	–	–	7 mm (9)	PR/7<
	lck 208	C	ArArA	–	+ (6)	–	10 mm (6)	
	lck 488	A	Ar	–	+ (9)	–	10 mm (6)	
2	SART3 109	B	–	–	+ (6)	–	–	SD/7
	lck 208	A	AAAA	–	–	–	–	
	lck 486	AB	A	–	+ (9)	–	–	
3	CyB 91	A	C	–	–	–	–	PD/3
	lck 488	A	A	–	–	–	–	
	ART4 75	A	–	–	–	–	–	
4	CyB 91	A	–	–	–	–	–	PD/3
	lck 208	A	C	–	–	–	7 mm (3)	
	lck 486	AAAA	C	–	–	–	7 mm (3)	
	lck 488	AA	–	–	–	–	7 mm (3)	
5	SART3 109	C	ArB	–	–	–	–	PD/4
	lck 208	C	A	–	+ (9)	–	–	
6	SART3 109	C	ArBC	–	+ (6)	–	–	PD/3
	ART4 75	B	B	–	–	–	–	
7	SART3 109	A	A	–	+ (6)	–	10 mm (3)	PD/4
	SART3 315	C	–	–	–	–	–	
	CyB 91	C	–	–	–	–	–	
	lck 208	C	C	–	–	–	–	
8	SART1 690	A	–	–	–	–	–	PD/4
	SART2 93	AB	–	–	–	–	–	
	SART2 161	A	C	–	–	–	–	
	lck 208	B	–	–	–	–	–	
9	SART1 690	AA	–	–	–	–	–	PD/4
	SART2 161	AA	–	–	–	–	–	
	SART3 109	A	–	–	–	–	–	
	lck 486	AB	C	–	+(6)	–	–	
10	SART3 315	C	ArAA	–	+(6)	–	–	PD/3
	CyB 91	A	–	–	–	–	–	
	lck 208	AAA	–	–	–	–	–	
	lck 486	AA	–	–	–	–	–	

[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 (armed response), $P<0.1$ and IFN-γ production $>500\,pg\,ml^{-1}$; A, $P<0.05$ and IFN-γ production $>50\,pg\,ml^{-1}$; B, $P<0.05$ and $50\,pg\,ml^{-1}$ >IFN-γ production $>25\,pg\,ml^{-1}$; C, $0.05<P<0.1$ and IFN-γ production $>25\,pg\,ml^{-1}$. The classification is shown by letters of the alphabet, and each character represents the results of each well. For example, ArBC means that three wells were judged as Ar, B, and C, and one well was negative. [b]The number in the parenthesis represents the vaccination when anti-peptide IgG or DTH was detected for the first time.

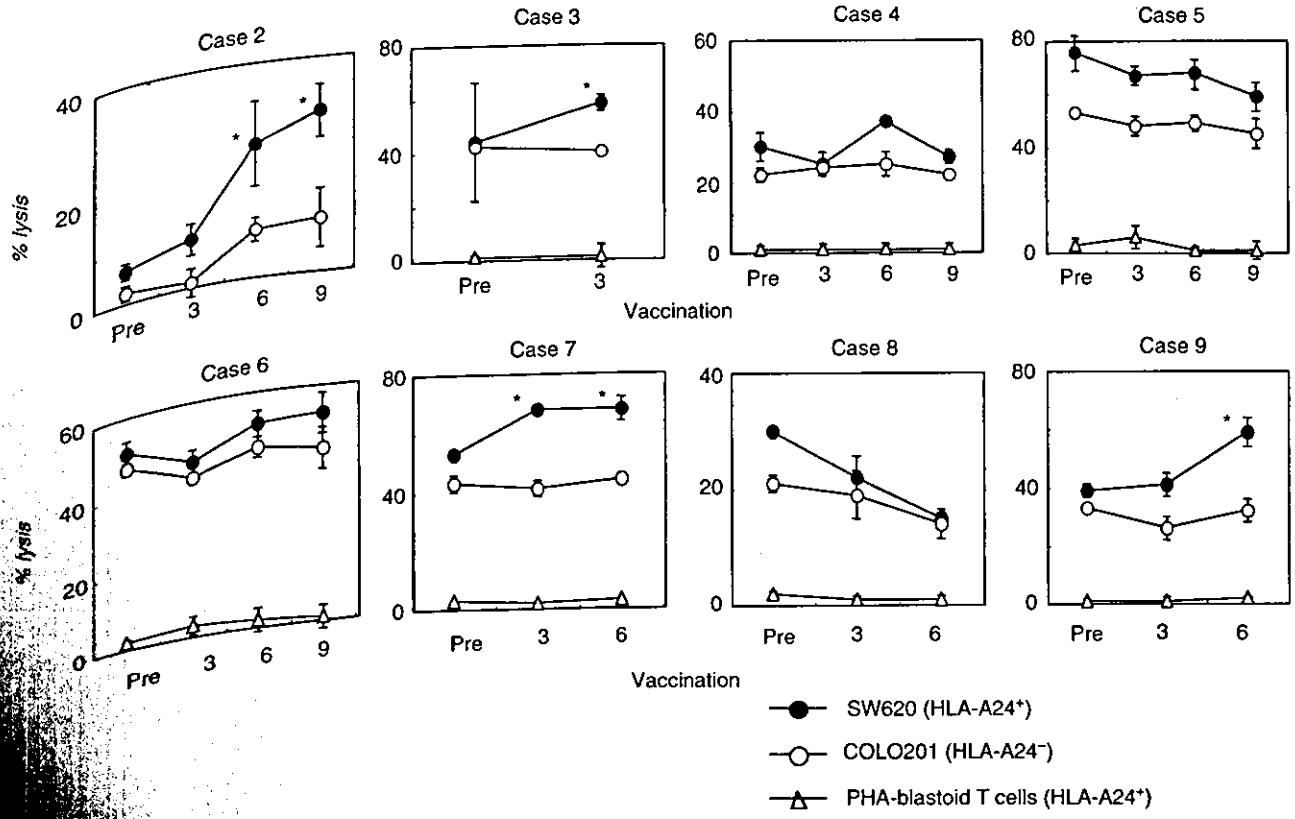
et al, 2000; Nakao *et al*, 2000; Nishizaka *et al*, 2000). As shown in four cases of patients 2, 3, 7, and 9, cytotoxicity against HLA-A24$^+$ SW620 increased after peptide vaccination compared to that against HLA-A24$^-$ COLO201. In the other four cases, no definite increase in cytotoxicity was observed after the peptide vaccination. No cytotoxicity against HLA-A24$^+$ PHA-blastoid T cells was detected in any case. These results indicate that the peptide vaccination resulted in augmented CTL activity in four out of eight patients.

Serum IgG specific to peptides

No IgG reactive to any of the vaccinated peptides was detected in pre-vaccination sera from any of the 10 patients (Table 4). Significant levels of anti-peptide IgG reactive to SART3- or lck-derived peptides became detectable in the post-vaccination sera of seven patients. Detail results are shown in Figure 3. In patient 1, IgGs reactive to the lck_{208} and the lck_{488} peptides were induced after the 6th and 9th vaccinations, respectively (Figure 3A). A similar result was observed in six other patients (Figure 3B). It is noteworthy that three patients who showed strong peptide-specific CTL response (criteria Ar) after the peptide vaccination were also positive for antipeptide IgG to the corresponding peptides (patient 1 for lck_{208} and lck_{488}, patient 6 for $SART3_{109}$, and patient 10 for $SART3_{315}$). In addition, the IgG response to the lck_{208} peptide in post-vaccination sera of patient 1 was neutralised by absorption with a corresponding peptide, but not with the lck_{408} peptide, whereas the opposite was observed in the case of the IgG response to the lck_{488} peptide (Figure 3C). This peptide-specific absorption demonstrates the validity of the ELISA system.

DTH skin test

No DTH reaction against peptides was observed before vaccination in any patient, while peptide-specific DTH reactions were observed



...cytotoxicity before and after the peptide vaccination. Pre- and post-vaccination PBMCs from eight patients were incubated for 14 days with IL-2 ... peptides in culture, followed by measurement of cytotoxicity against SW620 cells (HLA-A24$^+$ colon cancer cell line), COLO201 cells ... 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 ... and s.d. are shown. *Statistically significant at $P<0.05$.

...after the peptide vaccination (Table 4). In patient ... lck_{208} and lck_{488} were observed after the 6th ... DTH against $SART3_{109}$ became detectable after ... Patient 4 exhibited a DTH reaction against ... lck_{488} after the 3rd vaccination. Patient 7 ... reaction against $SART3_{109}$ after the 3rd

... of each patient are summarised in ... patient 1 pre- and post-vaccination are ... (In all, 48% regression (27–14 mm) of ... observed in patient 1. This patient had para-... metastasis at a diameter of less than 20 mm; it ... the peptide vaccination (data not shown). ... response was considered a partial response ... analysed more in detail. The kinetic analysis ... patient 1 indicates that increased CTL ... cells became detectable in post-vaccination ... cytotoxicity against lck_{208} peptide-loaded ... significant after the 3rd and 6th ...C). While no peptide-specific IFN-γ ... in any of the 96 wells containing ... vaccination PBMCs, $SART3_{109}$-specific ... detected in two, two, and four wells among ... vaccination (3rd, 6th, and 9th) PBMCs, ... Production of lck_{208}-specific IFN-γ ... in three and four wells among 96 wells ... 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

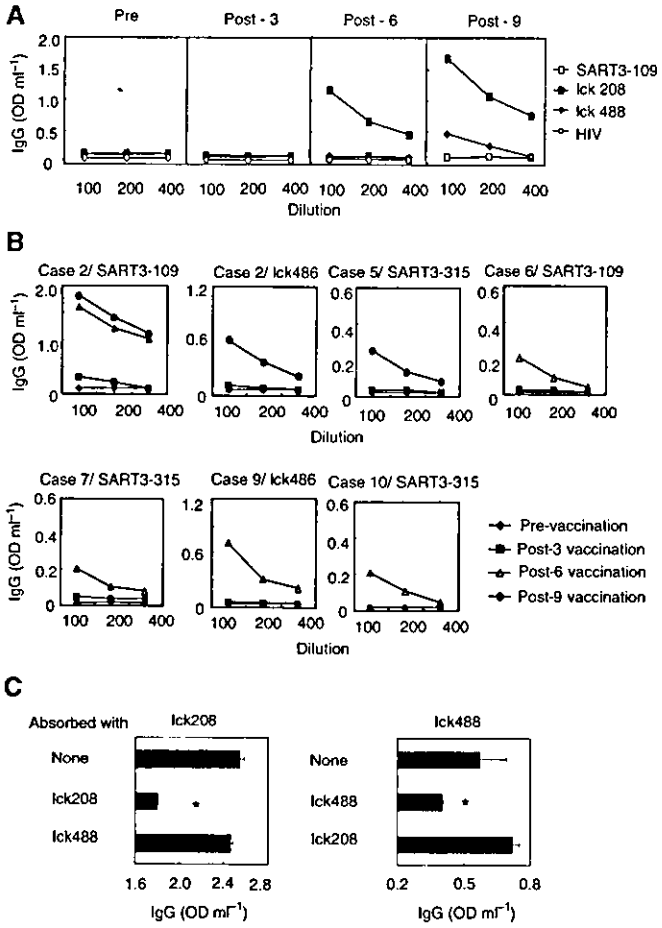


Figure 3 IgG reactive to the vaccinated peptides. (**A**) Pre- and post-vaccination sera from patient 1 were serially diluted and the levels of IgGs reactive to three administered peptides were determined by ELISA. (**B**) Pre- and post-vaccination sera from patients 2, 5, 6, 7, 9, and 10 were serially 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.

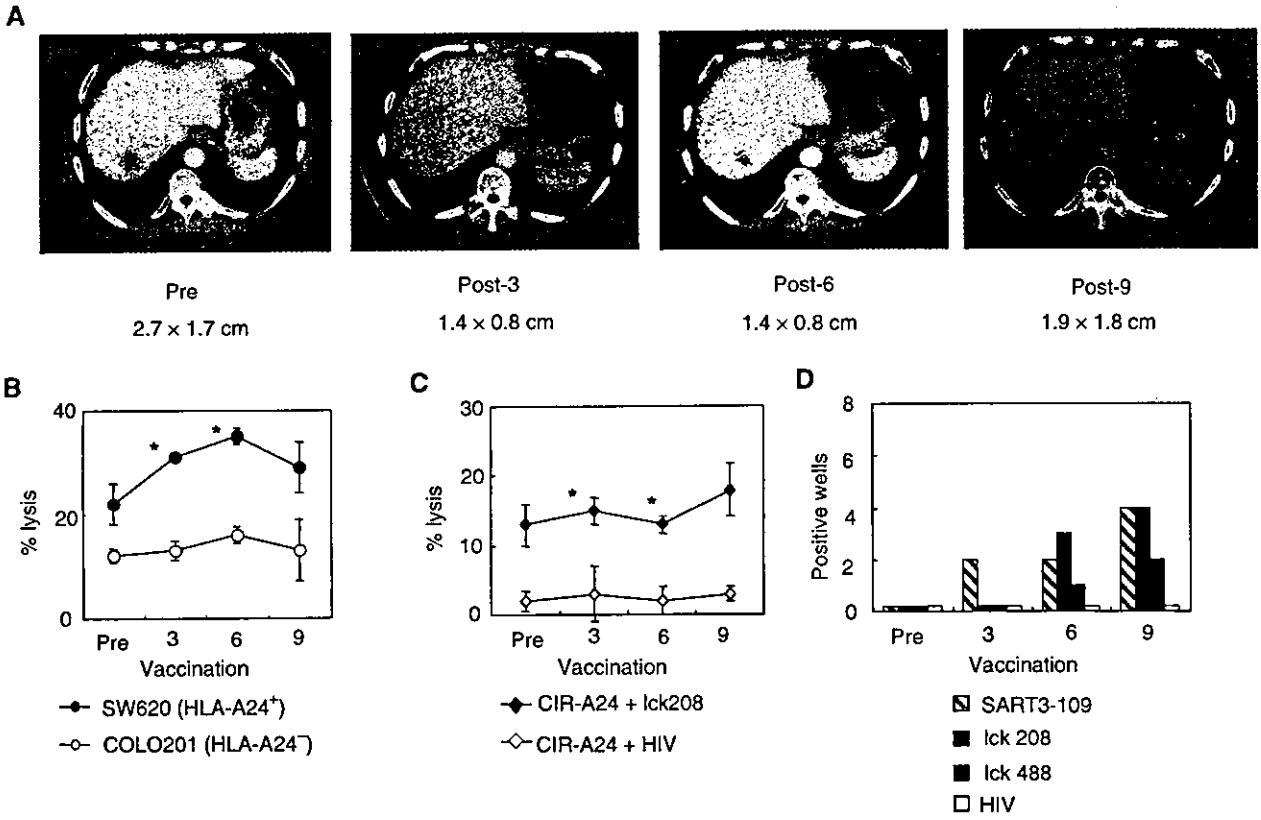


Figure 4 Clinical and immunological responses to the peptide vaccination. (**A**) CT scans show tumour regression of the liver metastasis after the peptide vaccination. The size of the liver metastasis (S8) is described. (**B**) CTL activity before and after vaccinations. Cytotoxicity to SW620 cells (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.

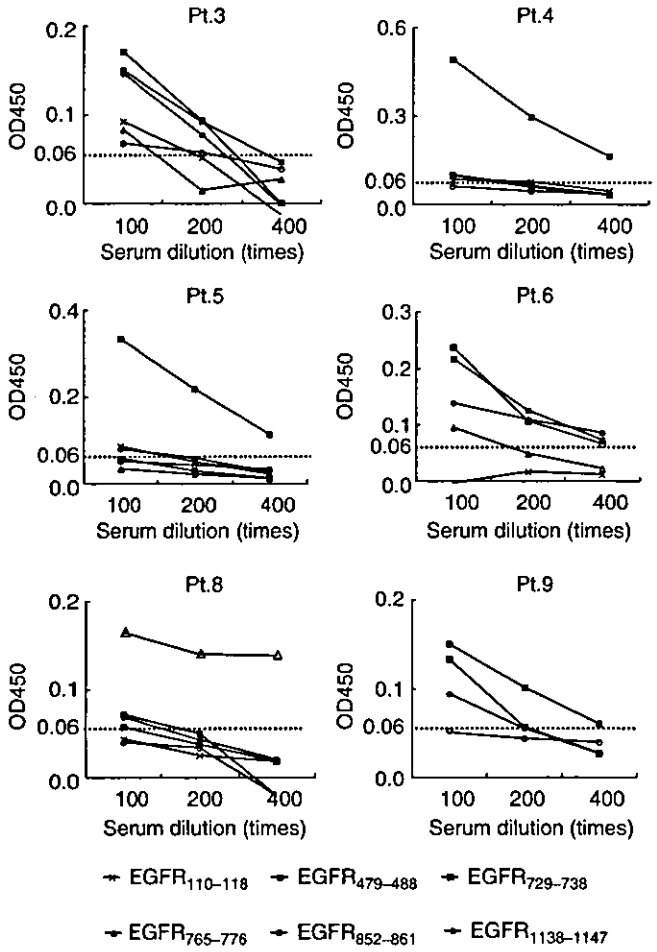

Figure 1 Detection of antipeptide IgG. Optical density values of each sample were assayed in serially diluted serum samples to estimate peptide-reactive IgG levels by the ELISA. The OD value against an irrelevant peptide (HIV) used as a negative control was subtracted from the data. Representative results 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

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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 —.

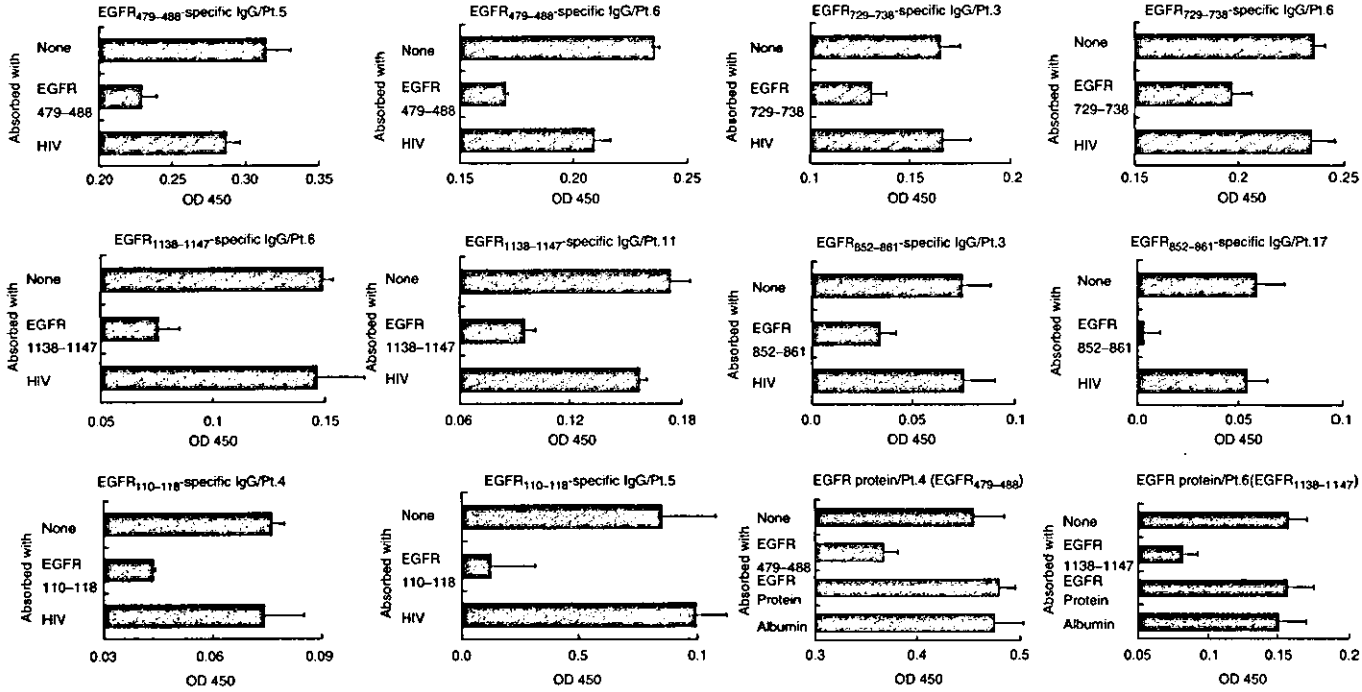

Figure 2 Specificity of antipeptide IgG. Each serum sample was absorbed with either a corresponding peptide or an HIV peptide used as a negative control three times at 37°C followed by testing of peptide-specific IgG activity with ELISA. Results of five peptides in the sera of each two representative 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.

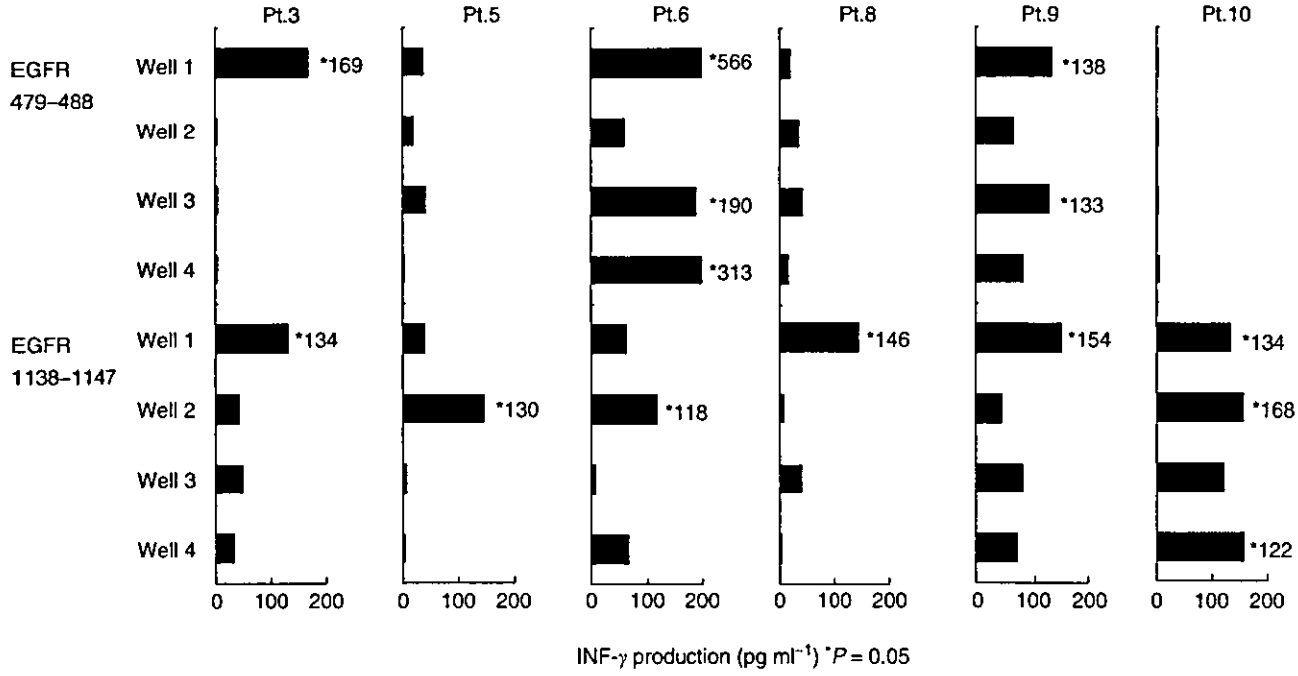

Figure 3 Cellular responses to peptide. Peptide-stimulated PBMCs from HLA-A2$^+$ cancer patients were cultured in four different wells (15×10^4 well^{-1}). On day 14 of culture, the peptide-stimulated PBMCs ($80-120 \times 10^4$ well^{-1}) from each well were independently collected and divided into 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

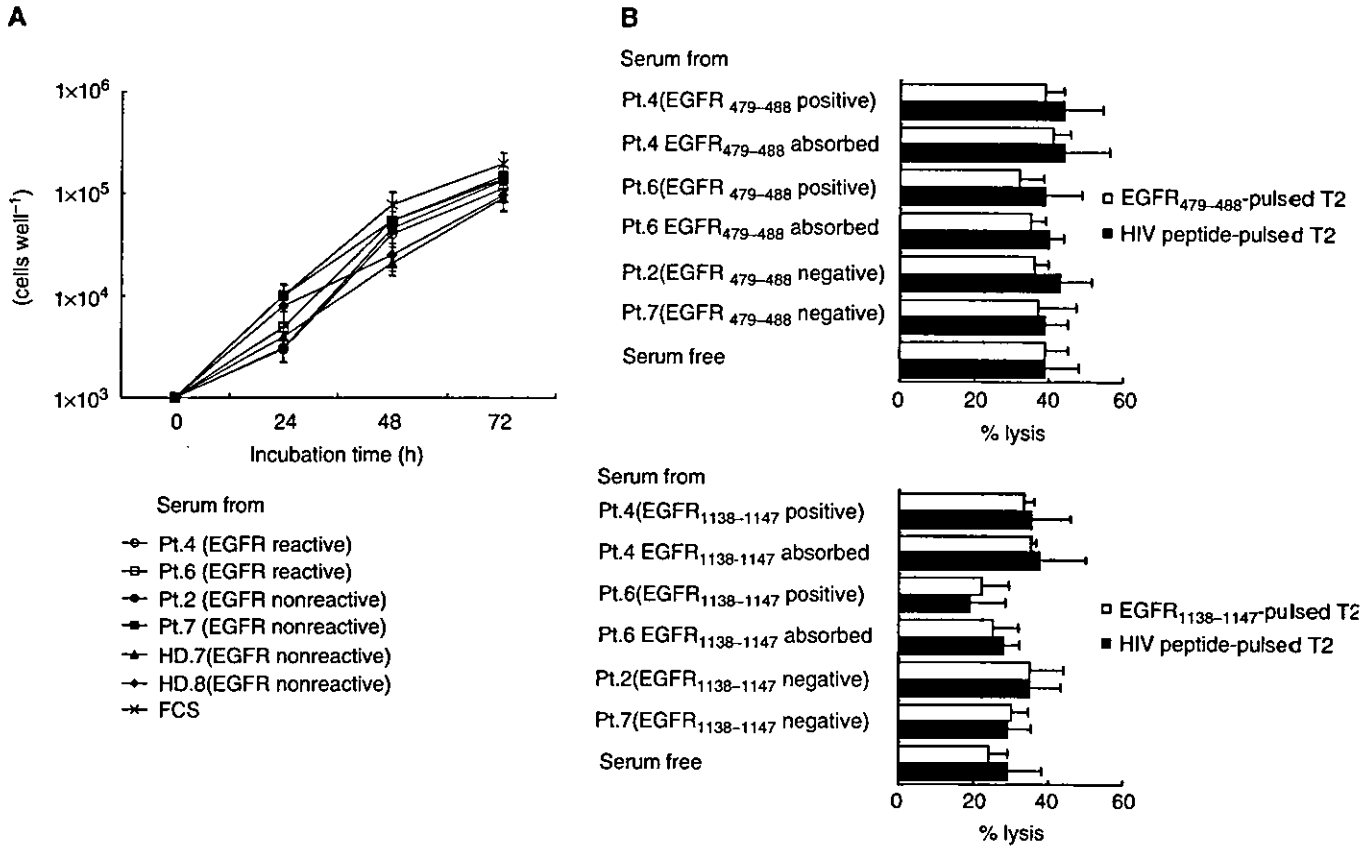

Figure 4 Direct inhibition and antibody-dependent cell-mediated cytotoxicity of sera possessing antipeptide IgG. (**A**) To test the direct growth inhibition activity of antipeptide IgG, 11–18 tumour cells were cultured in the presence of three different concentrations of sera, which had detectable levels 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