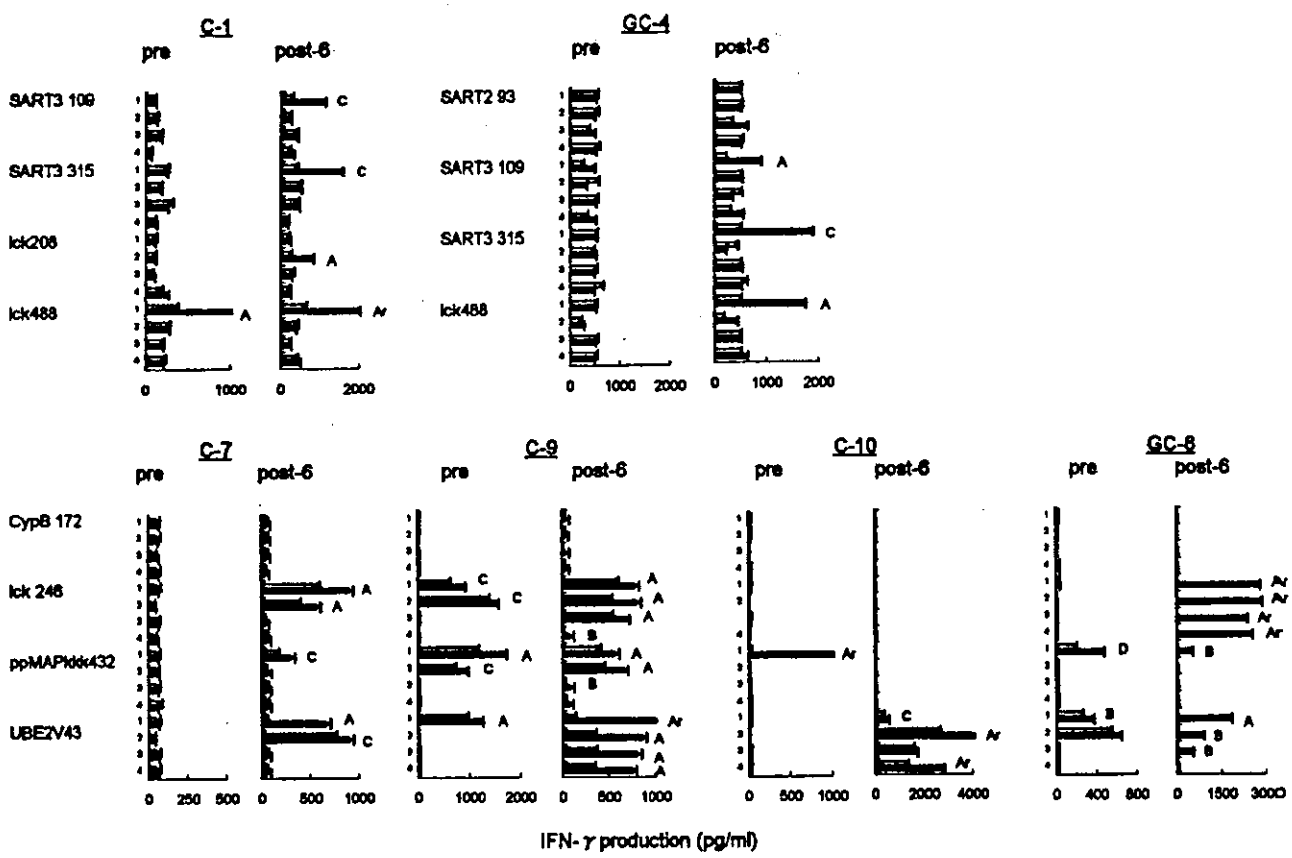

Figure 1. Cellular response to peptide. Pre- and post (6th)-vaccination PBMCs ($1x10^5$ cells/well) were incubated with 10 μM of each peptide in 4 different wells of a 96-well microculture plate in 200 μl culture medium containing IL-2, as reported previously (12). On culture day 14, the peptide-stimulated PBMCs ($8\text{-}12x10^5$/well) from each well were independently collected and divided into the four equal portions. Two such portions were separately tested for their ability to produce IFN-γ in response to C1R-A2402 cells for HLA-A24+ or T2 cells for HLA-A2+ PBMCs in the duplicate assays, while the remaining two portions were tested for a negative control peptide (HIV). Background IFN-γ production in response to the HIV peptide (<50 pg/ml) was subtracted. The representative results of 6 cases are shown. A detailed summary is provided in Table II. Criteria for evaluation of cellular responses are also described in the legend of Table II.

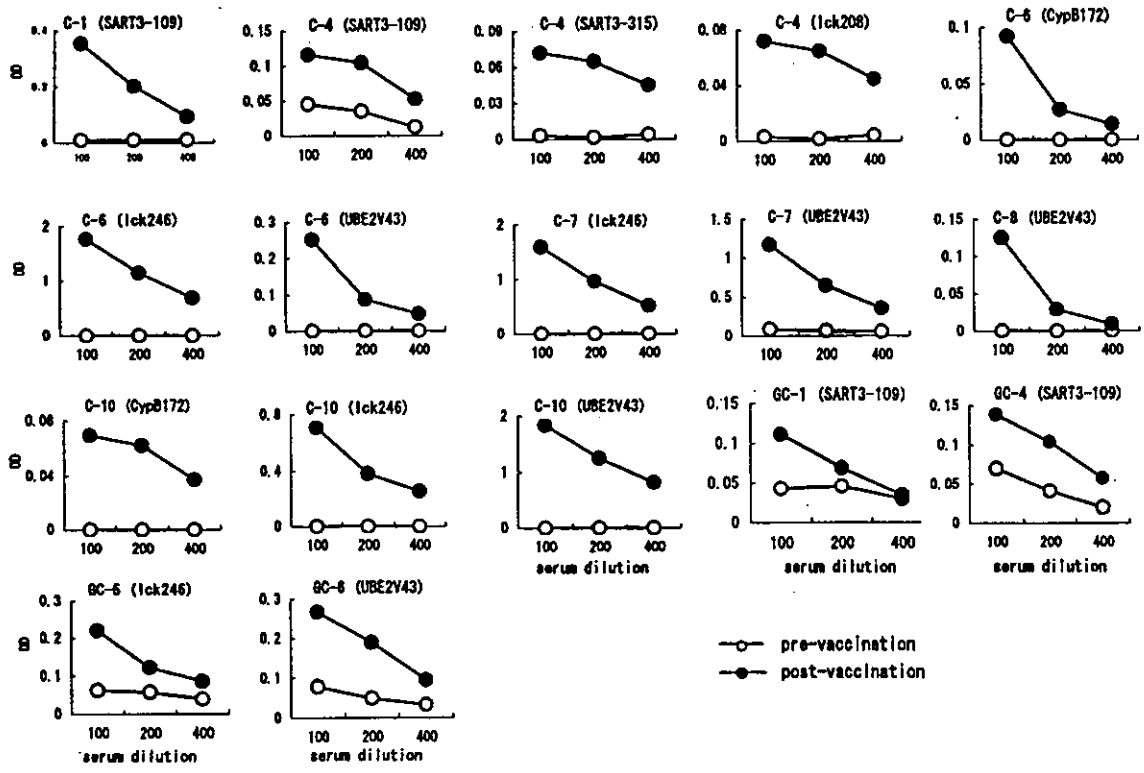

Figure 2. Serum IgG reactive to vaccinated peptides. Pre- and post (6th)-vaccination sera were provided for the assay, and all positive results for anti-peptide IgGs in 9 patients are shown. The peptide specificity of each of these IgGs has been previously described (6-12).

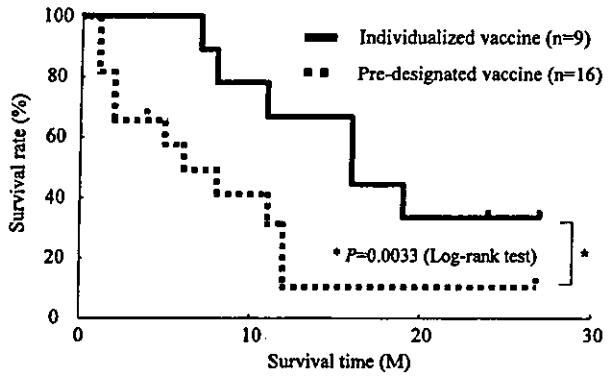

Figure 3. Survival time of the patients under the two vaccination regimens for cervical cancer and sccirhous type GC patients. Details of survival of the 9 patients (5 cervical cancer patients and 4 with sccirhous type GC) under the individualized vaccination regimens have been reported previously (11,12).

than 2 peptides were detected in the pre-vaccination PBMCs. Namely, CTL precursors to 3 of 4 vaccinated peptides were detectable in the pre-vaccination PBMCs of patient C-9, and CTL precursors to 2 of 4 vaccinated peptides were detectable in the pre-vaccination PBMCs of patients C-4 and GC-8.

Shorter survival of patients in the pre-designated vaccination regimen. In this study, we carried out a new trial of the 'pre-designated' regimen, in which cancer patients were vaccinated with peptides that were frequently selected as vaccine candidates in the preceding individualized regimen. However, the clinical response seemed to be inferior to that of the individualized regimen. Therefore, we compared the overall survival time of cervical and GC (scirrhous-type) patients under this pre-designated regimen (n=16) to that under the preceding individualized vaccination regimen (n=9, 5 with

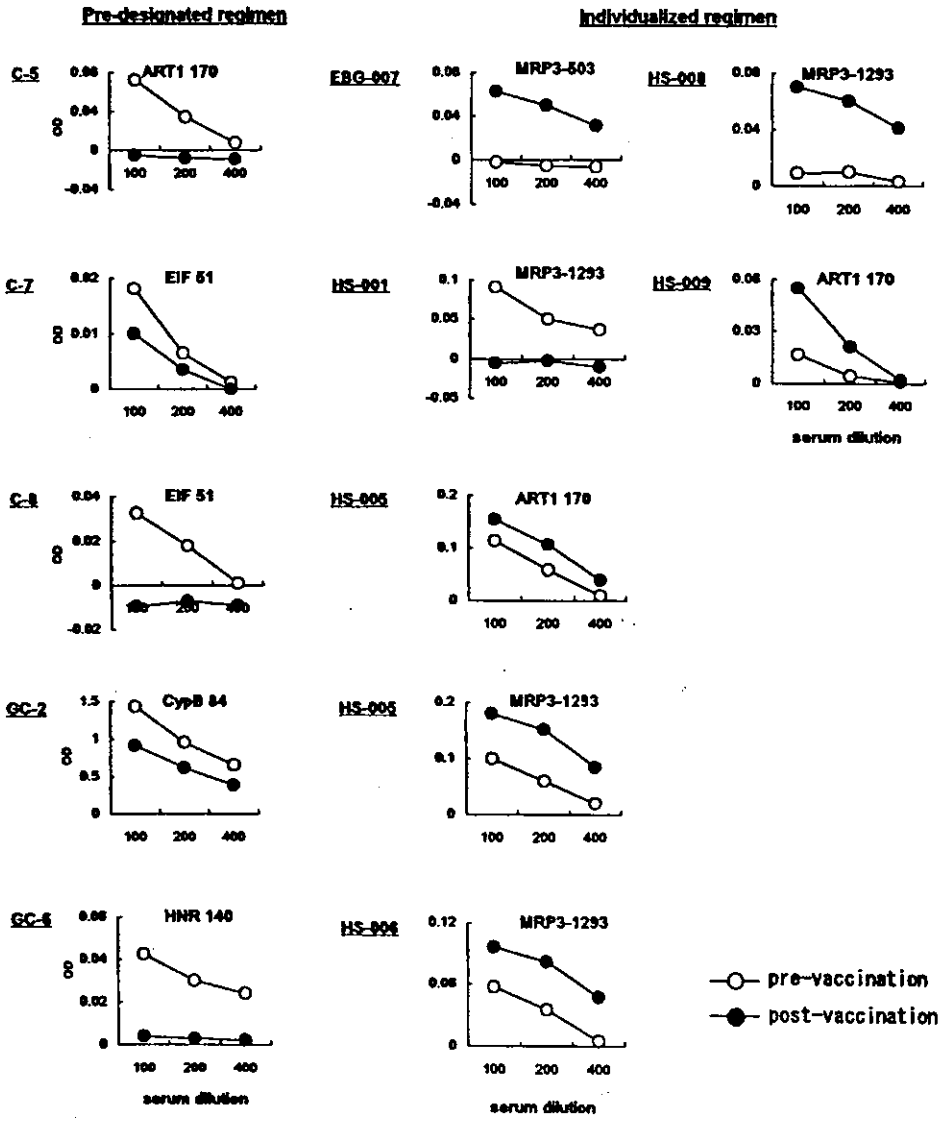

Figure 4. Serum IgG reactive to non-vaccinated peptides. Pre- and post (6th)-vaccination sera from the pre-designated vaccination regimen were provided for their reactivity to the non-vaccinated peptides, and the results from 5 cases whose post-vaccination sera showed decreased responses to the specific peptides are shown in the left column. Those from the individualized vaccination regimen were also provided for their reactivity to the non-vaccinated peptides, and the results from 6 cases whose post-vaccination sera showed increased or decreased responses to the specific peptides are shown in the right column. Detailed results for the individualized vaccinations were described elsewhere (11,12).

Table III. Immune response to peptides in the pre-designated and the previous individualized regimens.

Immune response	Reacted peptides/total peptides tested (%)		p-value[c]
	Pre-designated regimen[a]	Individualized regimen[b]	
DTH response	6/72 (8.3)	5/59 (8.5)	NS
Cellular response (IFN-γ)	26/52 (50)	9/48 (18.8)	<0.003
Humoral response (IgG) to vaccinated peptides:			
Increase	17/60 (28.3)	20/56 (35.7)	NS
Decrease	0/60 (0)	0/56 (0)	NS
Humoral response (IgG) to non-vaccinated peptides:			
Increase	0/83 (0)	6/50 (12)	<0.006
Decrease	6/83 (7.2)	1/50 (2)	NS

[a]Detailed results are given in Table II. [b]Detailed results are given in studies published previously (11,12). [c]χ^2 test was used for the statistical analysis. NS, not significant.

cervical cancer and 4 with scirrhous-type GC) (Fig. 3). As a result, the overall survival of the patients in this regimen was significantly (p=0.0033 by log-rank test) shorter than that of the preceding individualized regimen.

Incidental suppression of pre-existing immune responses by the pre-designated vaccination. As for the mechanism of the shorter survival of the patients in the pre-designated regimen, it is possible that, in the pre-designated regimen, pre-existing immune responses were incidentally suppressed by the induction of primary immune responses to the administered peptides for which CTL precursors did not exist. To test this possibility, we compared the level of IgG reactive to vaccinated peptides with that reactive to non-vaccinated peptides. The pre- and post (6th)-vaccination sera from 15 patients were tested for their levels of IgG specific to 6 different HLA-A24-restricted CTL epitope peptides ($CypB_{84}$, $ART1_{170}$, $ART4_{13}$, $ART4_{75}$, $MRP3_{503}$, and $MRP3_{1293}$) in HLA-A24$^+$ cases (n=8), and to 5 different HLA-A2-restricted CTL epitope peptides ($WHSC_{103}$, $WHSC_{141}$, $HNRPL_{140}$, $HNRPL_{501}$, and EIF_{51}) in HLA-A2$^+$ cases (n=7). None of the peptides were administered as vaccines in the pre-designated regimen, but all of them were administered in the preceding individualized regimen, and the peptide-specificity of serum IgG was reported elsewhere (8-14). The result was that the levels of peptide-specific IgG in the sera from 5 of 15 cases (C-5, -7, -8, GC-2, and -6) decreased after the pre-designated vaccination (Fig. 4). It is noteworthy that the TTPs of these 5 patients were very short (39, 41, 47, 53, and 65 days), and the median TTP was 43 days. On the other hand, in the individualized regimen, peptide-specific IgG increased after the vaccination with the exception of patient HS-001 with the $MRP3_{1293}$ peptide.

Immunological evaluation of two regimens. Finally, the immune responses to peptides in the pre-designated regimen and in the previous individualized regimen are summarized in Table III. There was no difference in DTH between the two regimens. However, cellular responses to 26 of 52 peptides (50%) tested were induced or increased in the post (6th)-vaccination PBMCs after the pre-designated regimen, while 9 of 48 peptides (18.8%) tested were induced or increased after the individualized regimen (p<0.003). This means that the pre-designated regimen is more potent than the individualized one. As for antibody responses, humoral responses to 17 of the 60 peptides (28.3%) tested increased in the post (6th)-vaccination sera with the pre-designated regimen, while 20 of 56 peptides (35.7%) tested increased with the individualized one. In both regimens, no decrease of IgG reactive to vaccinated peptides was observed. On the other hand, no induction of IgG reactive to non-vaccinated peptides was observed in the pre-designated regimen, whereas such IgG was observed in 6 of 50 peptides (12%) in the individualized regimen, and the frequency of the latter was significantly (p<0.006) higher than that of the pre-designated regimen. A decrease in the levels of IgG reactive to non-vaccinated peptides was observed in 6 of 83 peptides (7.2%) and in 1 of 50 peptides (2%) with the pre-designated and individualized regimens, respectively, although these levels of decrease were not significantly different from each other.

Discussion

One of the goals of tumor immunology is to develop a safe and therapeutically effective regimen of peptide vaccination for advanced cancer patients. However, no such regimen is available at the present time, despite the fact that many clinical trials of peptide vaccination regimens have been conducted in the past decade (6-12,15-21). To achieve this goal, we conducted this study to evaluate the safety, immunological and clinical responses of the pre-designated peptide vaccination of 4 peptides for which CTL precursors were found in pre-vaccination PBMCs at higher frequencies and were administered to patients as vaccines in the preceding regimen of the individualized vaccination (8-12). As a result, cellular and humoral responses to the vaccinated peptides were effectively induced.

The trial of the pre-designated regimen was essentially a prospective study of the previous individualized regime. In the pre-designated regimen, the levels of peptide-specific CTL precursors in the pre-vaccination PBMCs seemed to correlate with clinical outcome regarding both TTP and MST. In contrast, although the levels of either peptide-induced cellular or humoral immune responses were strongly induced in the post-vaccination PBMCs or sera of the majority of patients tested, they did not correlate with clinical outcome. These results may suggest that the individualized regimen is more suitable than the pre-designated regimen as a therapeutic vaccine for advanced cancer. This issue, however, should be carefully confirmed through relatively large scale clinical studies in the future, since, as far as our search of the literature revealed, all the past clinical trials of peptide-vaccination for cancer patients, with the exception of our recent trials (8-12), had been conducted as pre-designated regimens for advancer cancer patients (6,7,15-21).

The total doses of administered peptides for any given patient were different between the two regimens. The total doses during the first cycle of vaccination were 12 mg and 18 mg for the pre-designated (2 mg/week x 6 vaccinations) and individualized vaccination regimens (3 mg/2 weeks x 6 vaccinations), respectively. Suitable intervals of peptide vaccination remain to be determined. Therefore, as a pre-clinical study, we immunized Balb/c mice (H-2K^d mice sharing the peptide binding motifs with human HLA-A*2402) with the $SART3_{315}$ peptide on the two different schedules (2 mg/week x 6 vaccinations vs 3 mg/2 weeks x 6 vaccinations), then measured the immune responses to the $SART3_{315}$ peptide. As a result, mice of the former group showed much higher levels of peptide-specific response (Yamada *et al*, unpublished results). Based on this result, we conducted weekly administration of peptides on the pre-designated vaccination regimen, and compared to the results to those from the preceding individualized vaccinations (11,12).

There are no apparent differences between the two regimens with regard to patient characteristics, except for the numbers of cervical cancer patients (10 in the pre-designated regimen vs 5 in the preceding individualized regimen). Increased cellular responses to some of the vaccinated peptides were observed in the post-vaccination PBMCs of 11 of 13 patients (85%) and in 26 of the 52 peptides (50%) tested in this regimen, while they were observed in 11 of 18 patients (61%) and in 9 of the 48 peptides (18.8%) tested in the preceding individualized regimen (11,12). Humoral responses to peptides were observed in the post-vaccination sera of 9 of 15 patients tested (60%) and in 17 of the 60 peptides (28.3%) in the pre-designated regimen, while they were observed in 13 of 15 patients (87%) and in 20 of the 56 peptides (35.7%) in the preceding individualized regimen. These results indicate that the pre-designated regimen is superior to the individualized regimen with regard to the induction of the cellular responses to the administered peptides, whereas in the case of humoral responses the individualized regimen seems to be superior. However, a major difference in clinical response was observed between these regimens. Namely, the median TTP (n=16, 1.5 months) of patients with cervical cancer (n=10, 2 months) and scirrhous-type GC (n=6, 1 month) under this regimen was much shorter than that (n=9, 15 months) of patients with cervical cancer (n=5, 15 months) and scirrhous-type GC (n=4, 7 months) who underwent the preceding individualized regimen (11,12), respectively. The overall survival of these patients under this pre-designated regimen was significantly shorter than that under the preceding individualized regimen (Fig. 3).

The immunological mechanisms involved in the shorter survival associated with the pre-designated regimen are presently unclear. However, one possibility is that the induction of potent primary immune responses to the administrated peptides might suppress pre-existing specific immune responses to tumor cells. To test this possibility, we examined the levels of serum IgG reactive to CTL-epitope peptides. These levels were often found in both pre-vaccinated cancer patients and healthy donors, as reported previously (8-12,14,15). As a result, the levels of peptide-specific IgG in the pre-vaccination sera from 5 of 15 cases were found to be lower than those in the post-vaccination sera in association with the shorter TTP in these patients. With regard to peptides, the IgGs reactive to 5 among 11 kinds of peptides were found to be decreased. In contrast, such decreased humoral responses to non-vaccinated peptides were rarely found in the post-vaccination sera of patients under the individualized regimens.

In the present study, we assessed peptide-specific CTL responses based on a classification consisting of two parameters, the p-value and the amounts of IFN-γ release. The main reason for employing this classification was that the level of IFN-γ produced by peptide-specific CTLs varied among quadruplicate wells. Another reason was that we had to examine the presence of CTL precursors specific to different kinds of peptides using a limited number of PBMCs from cancer patients. Under these circumstances, cellular immune responses to substantial numbers of vaccinated peptides were not induced. This failure might be due in part to the limited sensitivity (1/3,000-1/10,000) as well as to relatively lower reproducibility of this CTL assay, which have been discussed elsewhere (8-12).

The increased levels of peptide-specific IgG induced by the individualized peptide vaccination correlated with the long survival of cancer patients in advanced stages of disease (10-12), whereas the IgG class has been found to be either lacking or unbalanced in atopic dermatitis patients (16), suggesting its role in host defense. However, the biological role of the peptide-specific antibodies in anti-tumor immunity is as yet unclear, and should be clarified by future basic and clinical studies. The mechanisms of peptide-specific IgG production, including the involvement of CD4$^+$ T helper cells and MHC restriction, also need to be clarified.

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Identification of epidermal growth factor receptor-derived peptides recognised by both cellular and humoral immune responses in HLA-A24$^+$ non-small cell lung cancer patients ☆

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Abstract

The epidermal growth factor receptor (EGFR) is one of the most appropriate target molecules for cancer therapy because of its high expression in epithelial cancers. A novel EGFR-tyrosine-kinase inhibitor, ZD1839, has been approved as a drug for non-small cell lung cancer (NSCLC), and many other agents are now being tested in clinical trials. Cytotoxic T lymphocyte (CTL)-directed epitope peptides could be another class of useful compounds in EGFR-targeted therapies. However, at present, there are no data on CTL-directed peptides of EGFR. Therefore, this study aimed to identify immunogenic EGFR-derived peptides in HLA-A24$^+$ NSCLC patients. We report in this study three such EGFR-derived peptides at positions 54–62, 124–132 and 800–809. These peptides were recognised by both cellular and humoral immune responses in most of the peripheral blood mononuclear cells (PBMCs) and sera from NSCLC patients that we tested. These results may provide a scientific basis for the development of EGFR-based immunotherapy.

Keywords: EGFR; Peptides; CTL; Ab; Lung cancer; Cancer vaccine

1. Introduction

The epidermal growth factor receptor (EGFR) plays an important role in epithelial biology and in many human malignancies [1–3]. Evidence that 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) [4,5]. In addition, cytotoxic T lymphocyte (CTL)-directed epitopes may also be useful in EGFR-targeted therapies as peptide vaccines for cancer patients whose tumours overexpress EGFR. However, there are no data indicating the CTL-directed epitopes of EGFR, although such peptides raised against HER2/neu, a member of the EGFR family, have been reported to be capable of inducing HLA-class I-restricted CTLs [6–9]. We previously reported that some CTL-directed peptides from non-mutated proliferation-related proteins had the ability to elicit both cellular and humoral im-

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mune responses *in vivo* in clinical studies [10–12]. Further more, levels of anti-peptide Abs in post-vaccination sera correlated with the overall survival rates of advanced cancer patients who had received peptide vaccinations [13]. In addition, there is evidence suggesting peptides have a high immunogenicity and are capable of inducing both cellular and humoral immune responses [14,15]. Therefore, with the view of developing peptide-based cancer therapies, we attempted in this study to identify peptides that might be vaccine candidates for HLA-A24$^+$ NSCLC patients.

2. Materials and methods

2.1. Samples and cell lines

Following written informed consent, sera and peripheral blood mononuclear cells (PBMCs) were collected from NSCLC patients at the Kurume University Hospital. PBMCs and sera were also obtained from healthy donors (HDs). All subjects were free from infection with the human immunodeficiency virus (HIV). Expression of HLA-class I antigens on these PBMCs was serologically defined by conventional methods as previously reported in [10]. Expression of EGFR in the tumour cell lines was examined by flow cytometric assay with an anti-EGFR monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA) [16], and representative histograms are shown in Fig. 1. Based on these results, the following tumour cell lines were used as target cells in a 6-h ^{51}Cr-release assay: 11-18 (HLA-A24/2, human lung adenocarcinoma, EGFR$^+$), QG56 (HLA-A26, lung squamous cell carcinoma (SCC), EGFR$^+$), Sq-1 (HLA-A24/11, lung SCC, EGFR$^\pm$), LC65A (HLA-A24/11, lung small cell carcinoma, EG- FR$^+$), SKOV3 (HLA-A3/28, ovarian cancer, EGFR$^+$) and SKOV3-A24 (HLA-A24-transfected SKOV3). Phytohaemagglutinin (PHA)-blastoid T cells from PBMCs were also used as a negative control for the target cells in the ^{51}Cr-release assay. For peptide loading, the C1R-A2402 (HLA-A2402 transfectant cell line) cell line was also used in this study.

2.2. Peptides and quantification of peptide-specific IgG

The following peptides were purchased from Bio-Synthesis (Lewisville, USA): 18 EGFR-derived peptides with HLA-A24 binding motifs at positions 43–51, 54–62, 68–76, 73–82, 111–119, 124–132, 269–277, 625–633, 722–730, 800–809, 812–821, 899–907, 899–908, 943–952, 960–969, 1015–1023, 1015–1024 and 1068–1077, respectively. An HIV peptide with a HLA-A24 binding motif (RYLRDQQLL) was also used as a negative control. Anti-peptide-specific IgG levels in sera were measured by an enzyme-linked immunosorbent assay (ELISA) as previously reported in [12,13]. The reactivities of sera from 10 HDs to an HIV peptide were measured by the assays and the sum of this mean value (0.02) and the standard deviation (SD) value (0.02) was set as the cut-off point (0.04). To test the specificity of the anti-peptide IgG in the sera, 100 μl/well of sera (100× dilution with 0.05% phosphate-buffered solution (PBS)) were absorbed with immobilised peptides (20 μg/well)

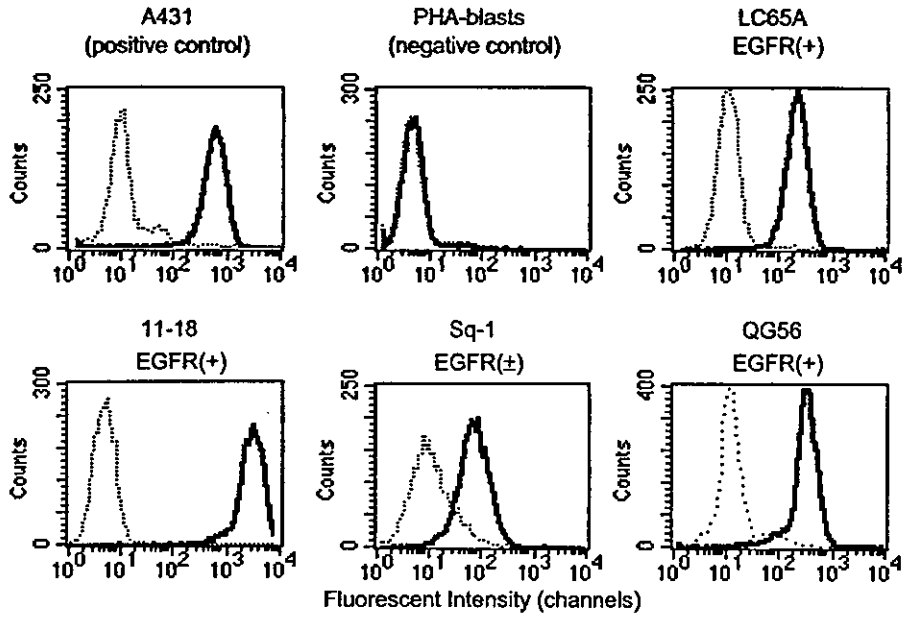

Fig. 1. Expression of epidermal growth factor receptor (EGFR) in tumour cell lines. A standard flow cytometric assay was used to detect EGFR expression on the tumour cells. The dotted line indicates the secondary antibody (Ab) alone. The black line indicates that anti-EGFR monoclonal Ab plus secondary Ab.

for 2-h at 37 °C, three times. Then, these absorbed sera were tested the anti-peptide IgG using an ELISA. To test the anti-peptide IgG response to the whole EGFR molecule, patients' sera containing anti-peptide activity were also absorbed with either immobilised EGFR protein (UPSTATE, Charlottesville, USA) or immobilised human albumin as a negative control, followed by measurement of the anti-peptide activity by ELISA.

2.3. Induction of cytotoxic T lymphocyte

PBMCs from HLA-A24$^+$ NSCLC patients and HDs served as subjects for the CTL induction assay. PBMCs (15×10^4 cells/well) were incubated with 10 μM of each peptide in a 96-well microculture plate (Nunc, Roskilde, Denmark) at a volume of 200 μl in culture medium containing 100 unit/ml of interleukin-2 (IL-2). Half of the culture medium was removed and replaced with the new medium containing a corresponding peptide (20 μM), every 3 days.

2.4. IFN-γ measurement using an ELISA and cytotoxicity analysis using the ^{51}Cr-release assay

These PBMCs as here were measured for peptide-specific interferon-2 (IFN-γ) production by the method previously described in [12]. In brief, cells in each well were separated into the two portions and co-cultured with a corresponding peptide or an HIV peptide for 14 days. After an 18-h incubation with C1R-A2402 cells pulsed with those peptides, the supernatant was collected and the level of IFN-γ was measured using duplicate ELISA assays. For each corresponding peptide, the assay was performed in the 4 independent wells. Then, cells that were 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 of ^{51}Cr-release assay was reported elsewhere in [12]. For the inhibition test, we used 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), anti-CD4 (Nu-Th/i, IgG1), and anti-CD14 (JML-H14, IgG2a) mAbs as a negative control. For a competition assay to study the peptide specificity of the cytotoxicity, unlabelled C1R-A2402 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.

2.5. ELISPOT analysis and CTL precursor analysis

PBMCs (5×10^4/well) from four patients, which had been cultured for 14 days by the methods described above, were further incubated for 24-h with the peptide-pulsed C1R-A2402 cells (1×10^5/well) in the sterile BD^{TM}ELISPOT plates coated with anti-human IFN-γ mAbs (BD Biosciences, San Diego, USA). The wells were then washed, coated with biotinylated anti-IFN-γ Ab (BD Biosciences), developed with avidin-alkaline phosphatase, stained and analysed using KS ELISPOT (Carl Zeiss, Hallbergmoos, Germany) [17]. These PBMCs from two patients were also provided for CTL precursor frequency analysis by the methods previously described in [18]. CTL precursor frequency was calculated using Taswell's method.

2.6. Statistics

The statistical significance of the data was determined by a two-tailed Student's t test and a P value of <0.05 was considered statistically significant throughout the study.

3. Results

We first investigated whether IgG reactive to each of the 18 EGFR-derived peptides could be detected in the sera of 13 NSCLC patients and 11 HDs. A summary of the results for 11 peptides, to which some of the sera showed a positive response, is given in Table 1 and representative results patients ((Pts) 2, 3 and 10, HD 2) are shown in Fig. 2. Significant levels of IgG (>0.04 OD values at a serum dilution of 100 times) reactive to the $EGFR_{800-809}$, $EGFR_{124-132}$ and $EGFR_{54-62}$ peptides were detected in the sera of 8, 7 and 6 of 13 patients, respectively. Sera from 9, 5 and 3 of 11 HDs tested also showed significant levels of IgG reactive to peptides $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$, respectively. In addition, significant levels of IgG reactive to the $EGFR_{899-908}$, $EGFR_{1015-1023}$, $EGFR_{269-277}$, $EGFR_{899-907}$, $EGFR_{812-821}$, $EGFR_{625-633}$, $EGFR_{73-82}$ and $EGFR_{1015-1023}$ peptides were detected in sera from one or two cancer patients, as well as in a few HDs. These humoral responses to EGFR peptides were observed in both HLA-A24-positive and -negative subjects, although most subjects in this study were HLA-A24-positive. In contrast, significant levels of IgG reactive to the remaining seven peptides were not detected in any of the sera tested (data not shown).

The peptide specificity of the anti-peptide IgG response to each of the $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$ peptides was confirmed using an absorption test (Fig. 3(a)). As a result, the activity of these sera reactive to each of the three peptides was absorbed with a corresponding peptide, but not with an HIV peptide used as a negative control. We also investigated using an absorption test whether the anti-peptide IgG reacts to the whole EGFR protein. However, the level of the anti-peptide IgG reactive to any of these three peptides was

Table 1
Humoral responses to EGFR peptides

Subjects	HLA	Responses to the EGFR peptides (OD values)[a]										
		$EGFR_{899-908}$	$EGFR_{1015-1024}$	$EGFR_{800-809}$	$EGFR_{269-277}$	$EGFR_{899-907}$	$EGFR_{124-132}$	$EGFR_{812-821}$	$EGFR_{625-633}$	$EGFR_{73-82}$	$EGFR_{54-62}$	$EGFR_{1015-1023}$
Pt. 1	A24/2	–[b]	–	–	–	–	–	–	–	–	0.05	–
Pt. 2	A24/33	–	–	0.13	–	–	0.05	–	–	–	0.05	–
Pt. 3	A24/2	–	–	0.14	0.06	–	0.07	–	–	–	0.07	–
Pt. 4	A2/11	0.05	0.16	0.06	0.05	0.05	0.05	0.05	0.05	–	0.05	0.06
Pt. 5	A24/31	–	–	–	–	–	–	–	–	–	–	–
Pt. 6	A2	–	–	–	–	–	–	–	–	–	–	–
Pt. 7	A1/24	0.13	–	–	–	–	–	–	–	0.06	–	–
Pt. 8	A24	–	–	0.05	–	–	0.49	–	–	–	–	–
Pt. 9	A24/2	–	–	0.05	–	–	–	–	–	–	–	–
Pt. 10	A24/11	–	–	0.09	–	–	0.13	–	–	–	–	–
Pt. 11	A24/2	–	–	–	–	–	–	–	–	–	–	–
Pt. 12	A24/2	–	–	0.13	–	–	0.08	–	–	–	0.15	–
Pt. 13	A24	–	–	0.13	–	–	0.12	–	–	–	0.14	–
HD 1	A24/33	0.08	0.09	0.08	–	–	–	–	–	–	–	–
HD 2	A24/26	–	0.20	0.16	0.10	–	0.10	–	–	–	0.06	–
HD 3	A2/26	0.22	1.50	0.20	0.18	0.34	0.09	0.15	0.05	–	0.13	0.66
HD 4	A24/26	–	–	0.09	–	–	0.05	–	–	–	–	–
HD 5	A2/24	–	–	0.08	–	–	–	–	–	–	–	–
HD 6	A11/33	–	–	–	–	–	–	–	–	–	–	–
HD 7	A2/24	–	–	0.05	0.05	–	–	–	–	–	–	–
HD 8	A31/33	–	–	–	–	–	–	–	–	–	–	–
HD 9	A2/24	–	–	0.19	–	–	–	–	–	–	–	–
HD 10	A2/11	–	–	0.11	–	–	0.05	–	–	–	–	–
HD 11	A2/24	–	–	0.18	–	–	0.07	–	–	–	0.14	–
Anti-peptides Abs	Pt. ($n = 13$)	2	1	8	2	1	7	1	1	1	6	1
	HD ($n = 11$)	2	3	9	3	1	5	1	1	0	3	1

ELISA, enzyme-linked immunosorbent assay; OD, optical density; EGFR, epidermal growth factor receptor; Pt., patient; HLA, human leucocyte antigen; HD, healthy donors.
[a] Anti-peptide IgG was assayed by ELISA as described in Section 2. Values represent the OD value at a serum dilution of 100 times.
[b] The OD values lower than the cut-off point (0.04) were shown as (–).

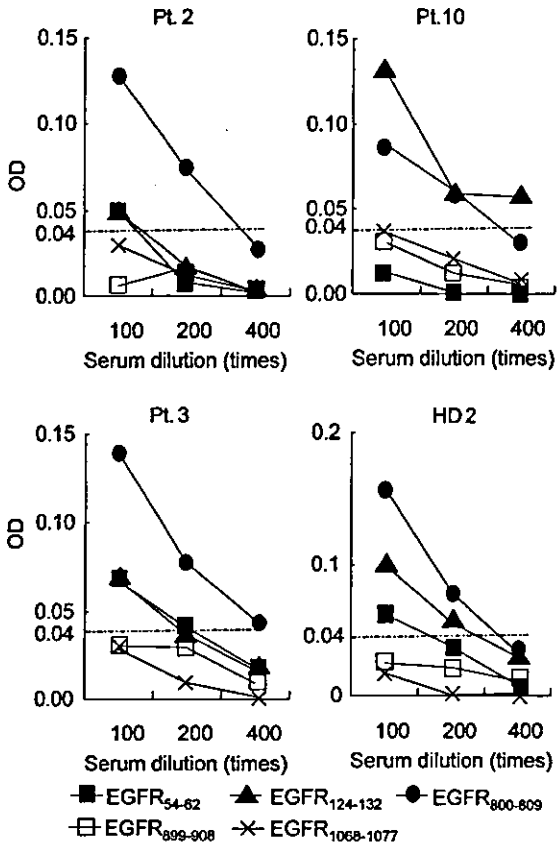

Fig. 2. Detection of anti-peptide IgG. The optical density (OD) value against a negative control human immuno defiencency virus (HIV peptide) was subtracted from the data. Representative results of patients (Pts) 2, 3, 10 and healthy donor (HD) 2 are shown.

not decreased at all by the absorption test (Fig. 3(b)), suggesting no cross-reactivity of the peptide IgG to the whole EGFR protein. Based on these findings, these three peptides were tested for their ability to induce CTL activity in PBMCs of HLA-A24$^+$ NSCLC patients and HDs in the following experiments. The two peptides ($EGFR_{43-51}$ and $EGFR_{943-952}$) to which no IgG response was detectable were also used as controls. Representative results of the four cases (Pts. 1, 11, 13 and HD 11) are shown in Fig. 4, and the summary of all subjects is given in Table 2. The $EGFR_{800-809}$, $EGFR_{54-62}$, and $EGFR_{124-132}$ peptides stimulated PBMCs from at least one of the four wells tested to produce statistically significant amounts of IFN-γ (P value of <0.05 and >100 pg/ml of IFN-γ) in response to C1R-A2402 cells pulsed with a corresponding peptide in 5, 5 and 4 of 8 cancer patients tested, respectively. These peptides also stimulated to produce significant levels of IFN-γ in 3, 4 and 4 of 5 HDs tested, respectively. The $EGFR_{43-51}$ and $EGFR_{943-952}$ also stimulated PBMCs to produce the significant levels of IFN-γ in 2 and 2 of 8 cancer patients tested, respectively (Table 2). The $EGFR_{43-51}$ and $EGFR_{943-952}$ stimulated PBMCs in 1 and 0 of 5 HDs tested, respectively.

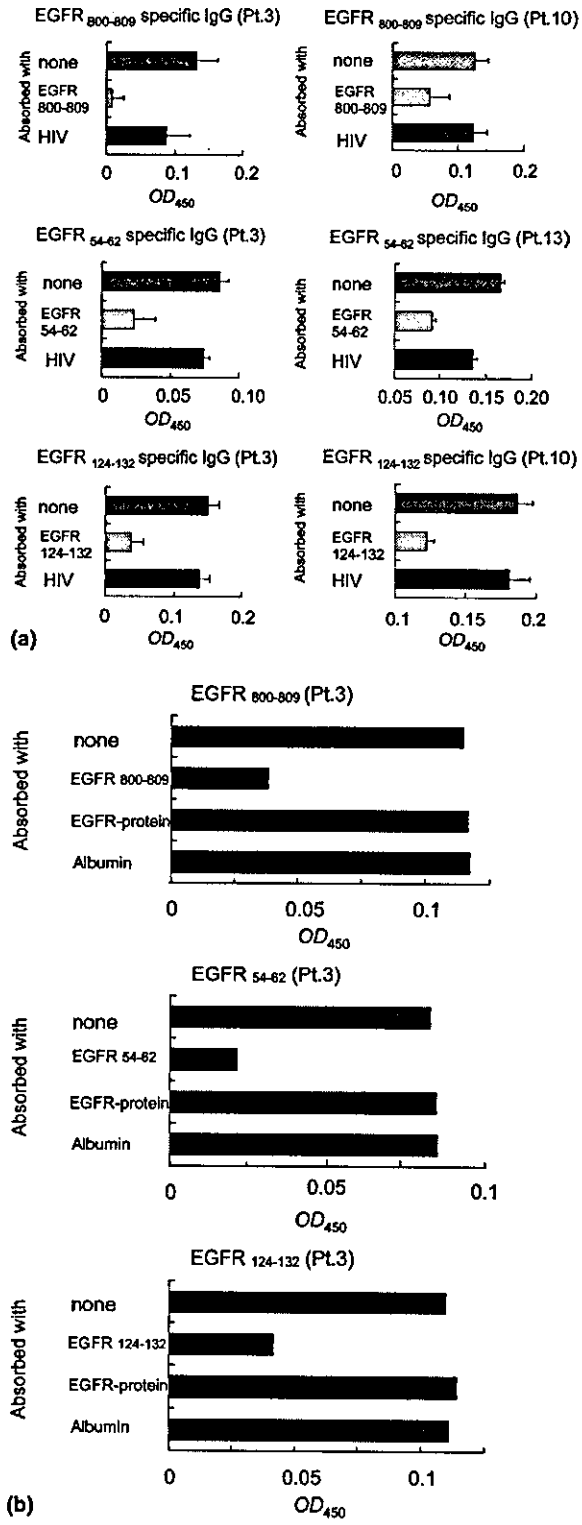

Fig. 3. Specificity of anti-peptide IgG: (a) absorption with either a corresponding peptide or an HIV peptide. The representative results from sera of Pts. 3, 10 and 13 are shown; (b) absorption with an EGFR protein or human albumin. The representative results from sera of Pt. 3 are shown.

To confirm the peptide-specific cellular responses measured by ELISA, we employed two different assays for the measurement of cellular responses; ELISPOT

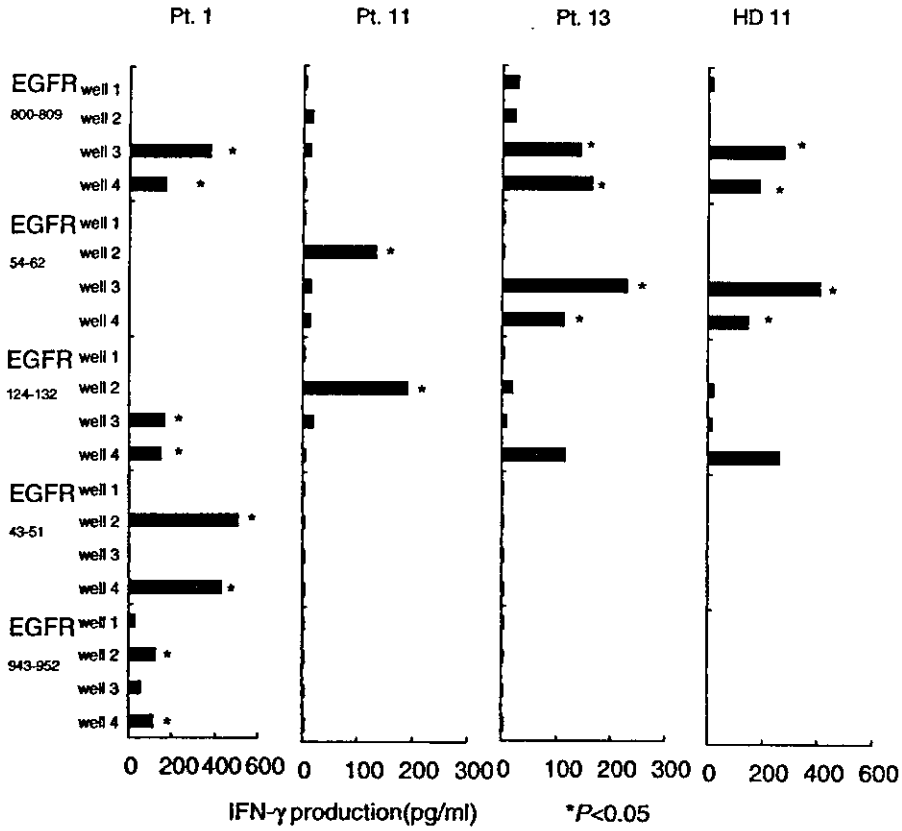

Fig. 4. Cellular response to peptide. The peptide-stimulated peripheral blood mono nuclear cells (PBMCs) were tested for their peptide-specific interferon (IFN)-γ production in quadruplicate assays. Background IFN-γ production in response to the HIV peptide (<50 pg/ml) was subtracted. * $P < 0.05$. The representative results of Pts. 1, 11, 13 and of HD 11 are shown.

Table 2
Cellular responses to EGFR peptides

Subjects	HLA	Responses to the EGFR peptides (IFN-γ production)[a]				
		$EGFR_{800-809}$	$EGFR_{54-62}$	$EGFR_{124-132}$	$EGFR_{43-51}$	$EGFR_{943-952}$
Pt. 1	A24/2	376/172	–[b]	168/150	509/432	126/115
Pt. 2	A24/33	–	–	138	–	–
Pt. 3	A24/2	430	154/170	–	160	–
Pt. 7	A1/24	572	116	–	–	122/376
Pt. 10	A24/11	–	–	–	–	–
Pt. 11	A24/2	–	134	192	–	–
Pt. 12	A24/2	122	202	–	–	–
Pt. 13	A24	166/144	231/115	118	–	–
HD 1	A24/33	–	110/116	166	130	–
HD 2	A24/26	161/863/184	316/314	1375/724	–	–
HD 4	A24/26	164	132/116	176/206	–	–
HD S	A2/24	–	–	–	–	–
HD 11	A2/24	280/190	410/150	267	–	–

PBMCs, pheripheral blood mononuclear cells; CTLs, cytotoxic T lymphocytes; IFN, interferon.

[a] PBMCs from HLA-A24+ lung cancer patients and HDs were stimulated with each of the indicated EGFR peptides. On day 14, the cultured PBMCs of the quadruplicate wells were tested for their reactivity to C1R-A2402 cells, which were pre-pulsed with a corresponding peptide in the duplicate assays. Values represent IFN-γ productions (pg/ml). Background IFN-γ production (<50 pg/ml) in response to an HIV peptide (taken as a negative control) was subtracted from the data shown in the Table. Successful induction of peptide-specific CTLs was judged to be positive when the supernatant of the well showed more than 100 pg/ml IFN-γ production with *P*-value of < at least 0.05. The mean values of amounts of IFN-γ of the positive wells among the 4 wells tested are shown in the table.

[b] (–) indicates when none of the 4 wells showed a positive response.

and CTL precursor frequency analyses. PBMCs from 4 NSCLC patients (Pts. 1, 3, 7 and 12) were at first provided for IFN-γ ELISPOT assays to further estimate the activity of the $EGFR_{800-809}$ and $EGFR_{54-62}$ peptides. An HIV peptide served as a negative control. As a result, $EGFR_{800-809}$ and $EGFR_{54-62}$ peptides had the ability to induce CTL activity in 3 and 3 of the 4 patients, respectively (Fig. 5(a)). As non-specific reactions, some positive spots in culture with an HIV peptide were observed in Pts. 1 and 7, although the numbers of spots were definitely lower than those induced by the $EGFR_{800-809}$ in Pt. 1 and the $EGFR_{54-62}$ peptide in Pt. 7. For the 6 positive cases, the ELISA also provided positive results, as shown in Table 2. There was no response in either assay for the $EGFR_{54-62}$ peptide in Pt. 1, whereas the $EGFR_{800-809}$-induced CTL activity in Pt. 7 was detectable by ELISA only. All these results suggest that peptide-specific CTL activity was detectable by either ELISA or ELISPOT assay in most cases. Fig. 5(a) also shows the humoral responses to the peptides. We investigated the peptide-specific CTL precursor frequencies (in Pt. 3 and 12) by stimulation with the $EGFR_{800-809}$ and $EGFR_{54-62}$ peptides. The CTL precursor frequencies reactive to $EGFR_{800-809}$, $EGFR_{54-62}$ and HIV peptides in Pt. 3 were 1/1329, 1315 and <1/10 000, while those in Pt. 12 were 1/976, 1/1662, and <1/10 000, respectively (Fig. 5(b)).

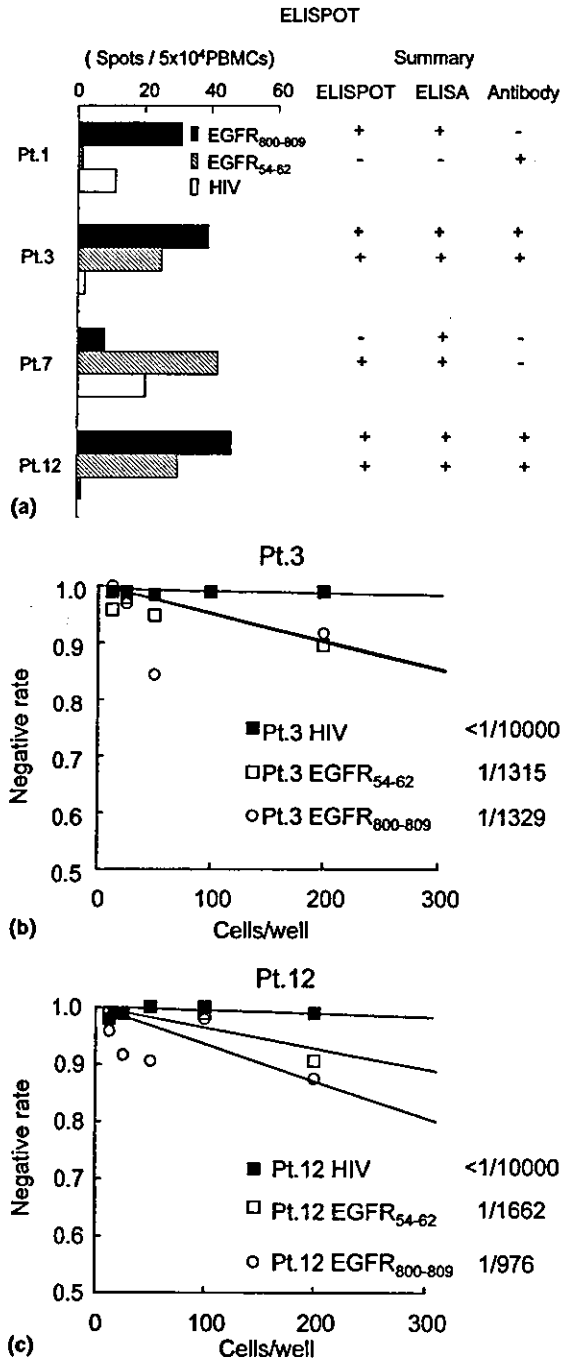

Fig. 5. IFN-γ ELISPOT and CTL precursor frequency: (a) peptide-stimulated PBMCs were tested for their peptide-specific IFN-γ production. The results of patients (Pts.) 1, 3, 7 and 12 are shown; (b) values represent the frequency of peptide-specific CTL precursor in Pts. 3 and 12.

The cytotoxicity of these peptide-stimulated PBMCs was confirmed by a 6-h ^{51}Cr-release assay, and representative results for 4 patients (Pts. 1, 2, 3 and 13) are shown in Fig. 6. These PBMCs showed significant levels of cytotoxicity against all the 11–18 NSCLC cells (HLA-A24$^+$, EGFR$^+$), LC65A small cell lung carcinoma cells (HLA-A24$^+$, EGFR$^+$) and SKOV3-A24 (HLA-A24$^+$, EGFR$^+$) tumour cells tested, but failed to kill any of the QG56 NSCLC cells (HLA-A24$^-$, EGFR$^+$), Sq-1 (HLA-A24$^+$, EGFR$^\pm$) NSCLC cells, or SKOV3 (HLA-A24$^-$, EGFR$^+$) tumour cells tested. These PBMCs also failed to kill PHA-blastoid T cells (HLA-A24$^+$, EGFR$^-$). PBMCs stimulated with an HIV peptide as a negative control did not show such HLA-A24-restricted cytotoxicity (Fig. 6, bottom left corner). These results suggest that these PBMCs possess HLA-A24-restricted cytotoxicity reactive to EGFR$^+$ tumour cells.

Further more, the restriction and peptide-specificity of the cytotoxicity were confirmed by inhibition and competition assays (Fig. 7). Namely, levels of cytotoxicity of these peptide-stimulated PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by the other mAbs tested in the assay. Cytotoxicity was also inhibited by the addition of the corresponding peptide-pulsed C1R-A2402 cells, but not by that of the HIV peptide-pulsed cells. These results suggest that the CTL activity was largely mediated by the peptide-reactive CD8$^+$ T cells in an HLA-class I-restricted manner.

4. Discussion

We reported in this study that the $EGFR_{800-809}$, $EGFR_{124-132}$ and $EGFR_{54-62}$ peptides were recognised

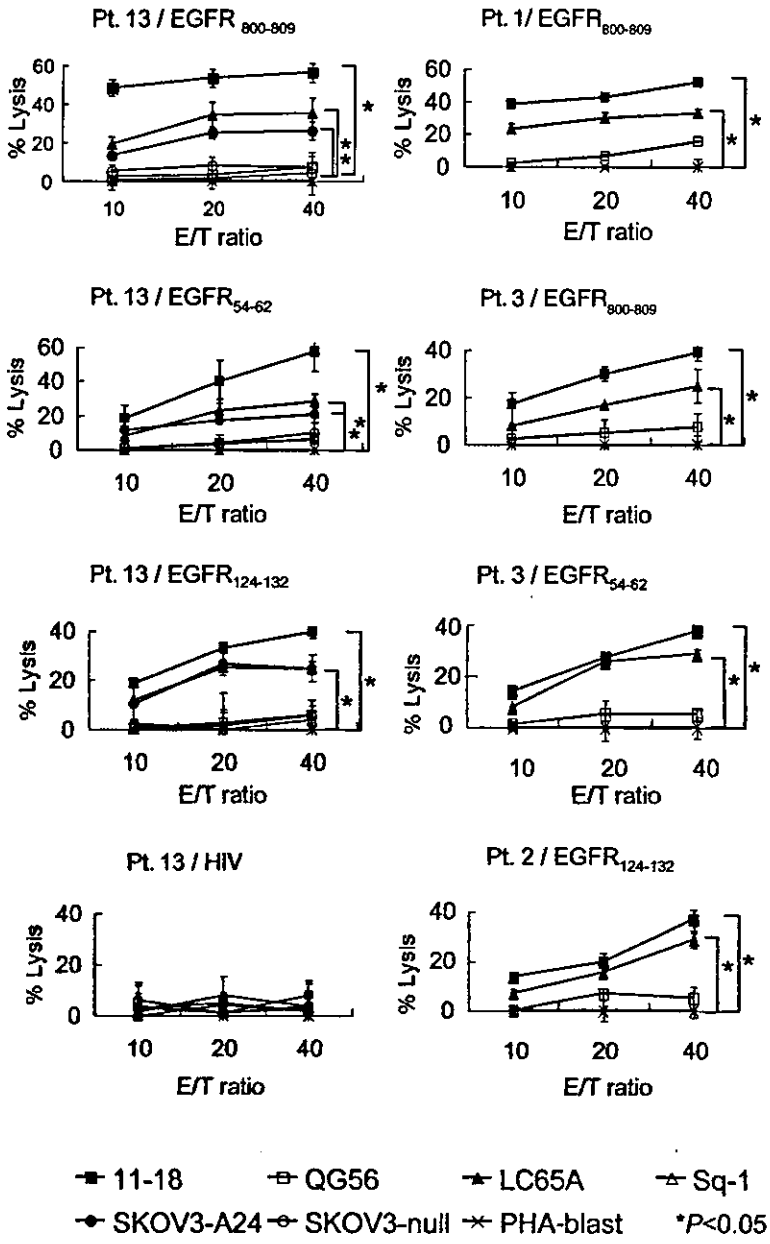

Fig. 6. Cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against various cancer cell lines using a standard 6-h ^{51}Cr-release assay. Representative results for Pts. 1, 2, 3 and 13 are shown. Values represent the mean ± standard deviation (SD) of % specific lysis. * $P < 0.05$.

by both cellular and humoral immune responses in >50% of PBMCs and sera, not only from NSCLC patients, but also from HDs. The immune responses in both cancer patients and HDs to EGFR peptides may not be surprising given that EGFR is expressed not only in epithelial cancer cells, but also in certain normal epithelial cells [1–3]. CTL precursors for peptides of HER2/neu, an EGFR family member, are also detectable in PBMCs from both cancer patients and HDs [6–9]. In addition to these three peptides, the $EGFR_{43-51}$ and $EGFR_{943-952}$ peptides also had the ability to induce peptide-reactive IFN-γ production in several NSCLC patients. However, their respective cytotoxicities were not investigated in this study, primarily due to the limited number of PBMCs available for the analysis. Cellular responses to the remaining 13 peptides with HLA-A24 binding motifs were also not investigated because of the limited number of PBMCs. Therefore, further studies will be needed to define the EGFR-derived peptides capable of inducing an HLA-A24-restricted cellular response alone.

It is generally accepted that ELISPOT and ELISA are well-established methods to measure cytokines, including IFN-γ, in the culture supernatants. Either assay is usually sufficient for measuring the CTL response if the ^{51}Cr-release assay, inhibition assay and peptide

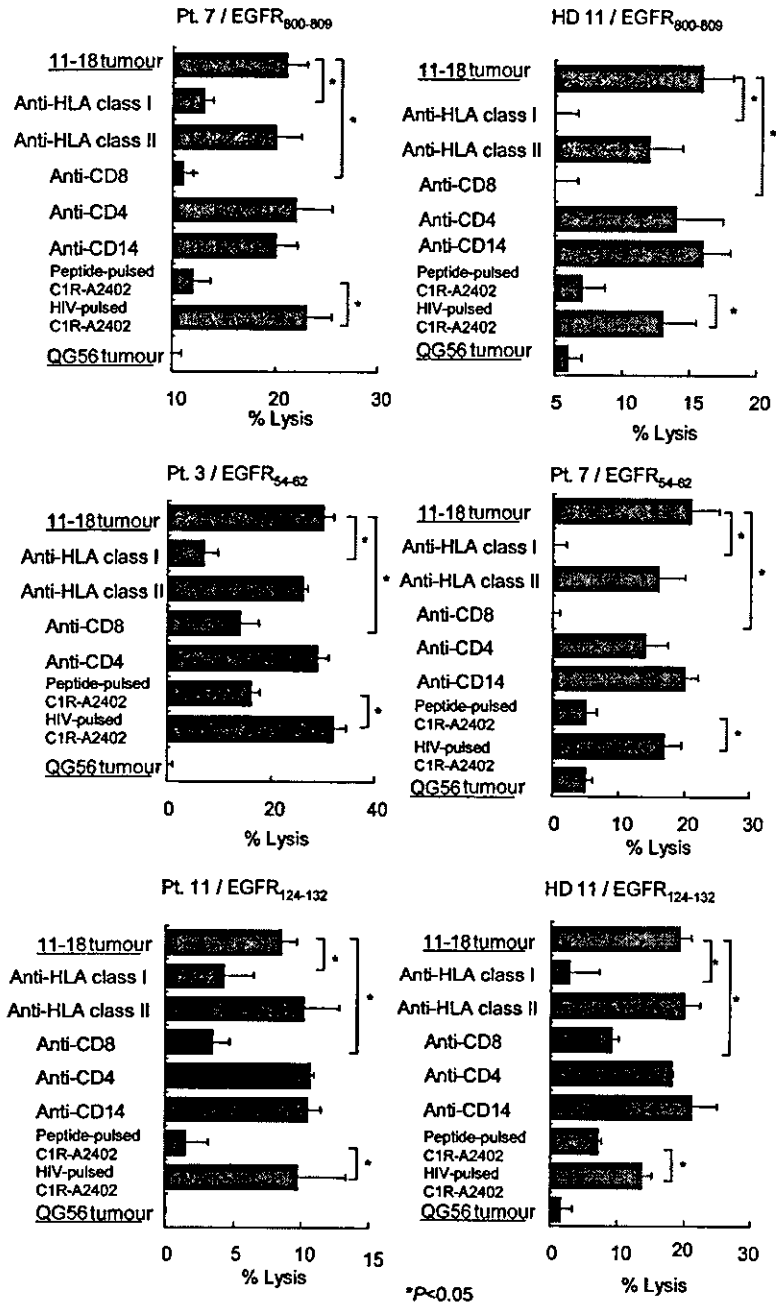

Fig. 7. Inhibition and competition assays. Peptide-stimulated PBMCs were tested for their restriction and peptide-specificity of cytotoxicity in the presence or absence of monoclonal antibody (mAb). For the competition assay, C1R-A2402 cells pulsed with the corresponding peptide or an HIV peptide were added 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 to 1. Values represent the mean ± SD of % specific lysis. *, $P < 0.05$.

specificity experiments are also conducted. To confirm whether the results obtained using an ELISA correlate well with those from other assays, we performed out both an IFN-γ ELISPOT and a classical CTL precursor frequency analysis in certain samples. As expected, the results obtained by these two assays correlated well with those from the ELISA.

We previously reported that IgG reactive against CTL epitope peptides was often detected in pre-vaccination sera of cancer patients and also in sera of HD, and there was no obvious HLA-class IA restriction involved [10–12,19,20]. Furthermore, 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 anti-peptide IgG in post-vaccination sera have correlated well with the overall survival rates of advanced cancer patients who received peptide vaccinations [13]. In contrast, IgG reactive to these CTL

peptides is either lacking or unbalanced in the sera of patients with atopic disease [20]. These results suggest that the IgGs to these peptides play a role in the host-defense response against these diseases, although the underlying mechanism of anti-tumour 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, are also presently unclear.

However, we showed that the peptide-specific IgGs did not react with the mother proteins at least for those we tested. In addition, sera containing anti-peptide IgGs failed to show direct growth inhibition effects and antibody-dependent cell-mediated cytotoxicity [21]. The anti-peptide IgGs may not act directly on tumour cells, but may facilitate the infiltration of immunocompetent cells into tumour sites through the induction of inflammatory reactions. This assumption is based on the fact that inflammatory reactions have been observed near tumours during surgery (radical prostatectomy) in prostate cancer patients who had entered into the peptide vaccination programme prior to undergoing prostatectomy (data not shown from our institute). Increased levels of IgG reactive to the vaccinated peptides were observed in the post-vaccination, but pre-surgery, sera of these prostate cancer patients.

As expected, positive cellular responses to peptides were not always associated with humoral responses. Namely, $EGFR_{800-809}$ and $EGFR_{124-132}$ peptides were recognised by both cellular and humoral responses in 6 and 5 subjects, whereas each peptide was recognised by either cellular or humoral responses alone in a few subjects. Similar results were observed for the $EGFR_{54-62}$ peptide, with a trend for a higher rate of cellular responses. One of the reasons for this discrepancy between cellular and humoral responses could be due to the differing sensitivities of the assays. Namely, the frequencies of circulating CTL precursors reactive to peptides are very low (less than 1/5000) [18] (data in this study), whereas Ig molecules (if they exist) are consistently detected in sera with a higher sensitivity. (if they exist). In addition, there are many biases affecting the detection of CTL precursors, including the condition of the frozen PBMCs and the culture conditions. Consequently, repeated experiments of CTL assays with different assay systems, if relatively large numbers of PBMCs are available, may increase the positive rate of peptide-specific cellular responses. It is also likely that a T cell response to a peptide will not always induce a B cell response in some subjects.

Although further studies are needed to clarify the biological role, as well as the mechanism of action of the peptide Abs, the three peptides recognised by both cellular and humoral immune responses can be presumed to be more immunogenic than those recognised by the cellular response alone. This assumption is, in part, supported by the fact that PBMCs of both cancer patients and HDs recognised each of the three peptides more frequently than they did either of the two peptides not recognised by serum IgG. The HLA-A24 allele is found in 60% of Japanese (with 95% of these cases being genotypically A2402), 20% of Caucasians and 12% of Africans [22]. Thus, these findings may provide new insight in to the development of EGFR-based immunotherapy that could be beneficial for substantial numbers of NSCLC patients through out the world.

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Expression of Epithelial Cancer-Related Antigens in Hematologic Malignancies Applicable for Peptide-Based Immunotherapy

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Abstract: Recent advances in tumor immunology have resulted in identification of many epithelial cancer-related antigens and peptides applicable to specific immunotherapy. The authors investigated whether these peptides, which are being studied clinically, could be appropriate target molecules for treatment of patients with hematologic malignancies. The majority of hematologic malignant cells studied expressed five different epithelial cancer-related antigens. Cytotoxic T lymphocyte (CTL) precursors reactive to these antigen-derived peptides were detected in peripheral blood mononuclear cells (PBMCs) of the majority of HLA-A24$^+$ patients, and the mean number of peptides recognized by CTL precursors was 2.4 per patient, ranging from 0 to 8 among the 10 peptides tested. These peptide-stimulated PBMCs exhibited HLA-A24-restricted cytotoxic activity against hematologic malignant cells but not against blastoid T cells. More importantly, these peptide-stimulated PBMCs exhibited cytotoxicity against freshly prepared autologous malignant cells in an HLA-A24-restricted manner. These results may provide a scientific basis for the use of these peptides from epithelial cancer-related antigens in specific immunotherapy for patients with hematologic malignancies.

Key Words: immunotherapy, peptide vaccine, hematologic malignancies

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Regarding immunotherapy against hematologic malignancies, several treatment modalities have been proposed: vaccination with irradiated autologous leukemia cells, bcl/abl chimera antigen, and immunoglobulin (Ig)-derived peptides.[1–4] Although several studies have reported major tumor regression in certain cases,[3–6] most patients fail to respond to these therapies, and the clinical benefit is limited at the present time. This failure could be in part due to the limited number of antigens and peptides for specific immunotherapy for patients with hematologic malignancies.

We previously identified a panel of epithelial cancer-related antigens, including SART-1, SART-2, SART-3, ART-1, and p56^{lck}.[7–11] SART-1, SART-2, and SART-3 antigens are expressed both in the nucleus of the majority of malignant tumor cell lines and cancer tissues and in the cytoplasm of proliferating cells. None of them, however, are expressed in normal tissues except for the testis and fetal liver.[7–9] ART-1 is expressed in the nucleus of malignant cell lines and proliferating cells, whereas its expression in normal tissues is limited to thymus, testis, and placenta.[10] p56^{lck}, the src family tyrosine kinase that is essential for T-cell development and function, is aberrantly expressed in metastatic epithelial cancers.[11] In addition, several lines of evidence suggest that p56^{lck} might contribute to the process of neoplastic transformation.[11] We also showed that peptides from these antigens have the potential to induce peptide-specific and tumor-reactive cytotoxic T lymphocytes (CTLs) in cancer patients.[7–11] Furthermore, we have used these peptides to vaccinate patients with various types of cancers, and enhanced immune and clinical responses were observed in some cases.[12,13]

In the present study, we determined whether the five epithelial cancer-related antigens could be appropriate target molecules for immunotherapy of patients with hematologic malignancies. We demonstrated that most hematologic malignant cell lines expressed these epithelial cancer-related antigens and that the peptide-stimulated peripheral blood mononuclear cells (PBMCs) from patients with hematologic malignancy showed HLA class I-restricted cytotoxicity against hematologic malignant cells in a peptide-specific manner.

MATERIALS AND METHODS

Clinical Materials

All clinical materials from patients with hematologic malignancies and healthy donors used in this study were ob-

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tained after the provision of written informed consent. None of the participants was infected with human immunodeficiency virus (HIV). Twenty milliliters of peripheral blood was obtained, and PBMCs were prepared by Ficoll-Conray density gradient centrifugation. All samples were cryopreserved until use for experiments. The expression of HLA-A24 molecules was determined by flow cytometry, as reported previously.[9]

Tumor Cell Lines

The following hematologic malignant cell lines were used in this study. REH, NALL1, NALM6, NALM16, KOPN-K, BALM1-2, and BALL-1 were B-cell acute lymphocytic leukemias (B-ALLs). KOPT, RPMI-8402, CCRF-CEM, HPB-ALL, MOLT-4, CCRF-HSB-2, and PEER were T-cell acute lymphocytic leukemias (T-ALLs). ML1, ML2, ML3, and KG1 were acute myelogenous leukemias (AMLs). THP-1 and U-937 were acute monocytic leukemias (AMOLs). RAJI and NAMALWA were Burkitt lymphomas. NALM1 and K562 were chronic myeloid leukemias (CMLs). ARH-77, U-266, KHM-11, MIK-1, and RPMI-8226 were multiple myelomas (MMs). BHL-89, HuT-102, and HEL were lines of B-cell lymphoma, T-cell lymphoma, and erythroleukemia, respectively. HPB-MLT was an adult T-cell leukemia (ATL). All cell lines were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS).

Immunostaining

The expression of SART-1, SART-2, and $p56^{lck}$ proteins in the cell lines was detected by Western blot analysis using anti-SART-1 800 polyclonal antibody,[7] anti-SART-2 polyclonal antibody,[9] and anti-$p56^{lck}$ monoclonal antibody (Lck3A5; Santa Cruz Biotech, Santa Cruz, CA), respectively. The expressions of SART-3 and $p56^{lck}$ proteins were analyzed by flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA). For intracellular staining, cells were pretreated with 4% paraformaldehyde and 0.1% saponin before the addition of anti-SART-3 monoclonal antibody[14] and anti-$p56^{lck}$ monoclonal antibody. FITC-conjugated anti-mouse IgG (ICN Biomedicals, Aurora, OH) was used as a secondary antibody.

Northern Blot Analysis and RT-PCR

The mRNA expression of the *ART-1* gene in the cell lines was examined by Northern blot analysis, as described previously.[10] Human β-*actin* cDNA (Clontech, Palo Alto, CA) was also used as a control probe. For reverse transcriptase–polymerase chain reaction (RT-PCR), total RNA was extracted from cells using RNA-Bee (TEL-TEST, Friendswood, TX). The cDNA was prepared using the Superscript first-strand synthesis system (Invitrogen). Two promoters of the *lck* gene were detected by the RT-PCR method using specific primer pairs, as reported previously.[11] PCR was performed using Taq DNA polymerase in a DNA cycler (iCycler; Bio-Rad Laboratories, Hercules, CA) for 30 cycles of 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 1 minute.

Peptides

All peptides (>95% purity) used in the in vitro study were purchased from Sawady Laboratory (Tokyo, Japan) and dissolved with dimethyl sulfoxide at a dose of 10 mg/mL. All peptides, with the exception of the Epstein-Barr virus (EBV)-derived and HIV-derived peptides, were derived from tumor-related antigens and have the ability to induce tumor-reactive and HLA-A24-restricted CTLs from PBMCs of cancer patients, as reported previously.[7–11] EBV-derived and HIV-derived peptides were used as positive and negative controls with an HLA-A24-binding motif, respectively.[15]

Assay for Peptide-Specific CTLs in PBMCs

The method used for the detection of peptide-specific CTLs has been reported elsewhere.[15] In brief, PBMCs (1×10^5 cells/well) were incubated with 10 μmol/L of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μL culture medium. The medium consisted of 45% RPMI-1640, 45% AIM-V (Gibco BRL), 10% FCS, 100 U/mL of IL-2, and 0.1 μmol/L MEM nonessential amino acid solution (Gibco BRL). On days 3, 6, and 9, half of the medium was removed and replaced with the new medium containing the corresponding peptide (20 μmol/L). On day 12 of culture, the harvested cells were tested for their ability to produce IFNγ in response to HLA-A24-expressing C1R cells, designated as C1R-A24, which were preloaded with either a corresponding peptide or the HIV peptide (RYLRQQLLGI), taken as a negative control. All assays were performed in quadruplicate, and cultured cells in one well were divided into four wells. Thereafter, two wells were used for control HIV peptide-pulsed cells, and the other two wells were used for the corresponding peptide-pulsed cells. Thus, cytokine release was assessed in two different ELISA wells. The level of IFNγ was determined by ELISA. A two-tailed Student *t* test was used for the statistical analysis. For the inhibition assay, the peptide-reactive CTLs were positively purified using a CD8 Isolation Kit (DYNAL, Oslo, Norway), and their peptide-specific IFNγ production was measured in the presence of 20 μg/mL of anti-HLA class I (W6/32, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a), anti-CD14 (JML-H14, IgG2a), or anti-HLA-A24 (A11.1, IgG3) monoclonal antibodies, as reported previously.[15] The peptide-stimulated PBMCs were further expanded in the presence of interleukin (IL)-2 (100 U/mL) alone for more than 14 days. The cultured cells were tested for cytotoxicity against various target cells by a 6-hour ^{51}Cr-release assay, as previously reported.[15] The specificity of the peptide-stimulated CTLs was confirmed by a cold target inhibition assay. In brief, ^{51}Cr-labeled target cells (2×10^3 cells/well) were cultured with the CTLs (4×10^4 cells/well) in 96 round-well plates with 2×10^4 cold target cells. C1R-A24 cells, which

were prepulsed with either the HIV peptide or a corresponding peptide, were used as cold targets.

RESULTS

Expression of Epithelial Cancer-Related Antigens in Hematologic Malignancies

We first studied the expression in a panel of hematologic malignant cell lines of the five tumor antigens (SART-1, SART2, SART-3, and p56^{lck} proteins, cloned from cDNA of a KE4 esophageal cancer cell line, and ART-1, cloned from a 11-18 lung adenocarcinoma cell line). The Western blot analysis revealed that the SART-1 antigen was expressed in all hematologic malignant cell lines tested (Fig. 1A). The KE4, taken as a positive control, expressed the SART-1, whereas no expression was observed in PBMCs, taken as a negative control. The Western blot analysis was also carried out to examine the expression of the SART-2 antigen in a panel of hematologic malignant cell lines. The SART-2 was expressed in all cell lines tested, whereas its expression was negative in PBMCs and phytohemagglutinin (PHA)-blastoid T cells (see Fig. 1B). The expression of the SART-3 antigen was analyzed

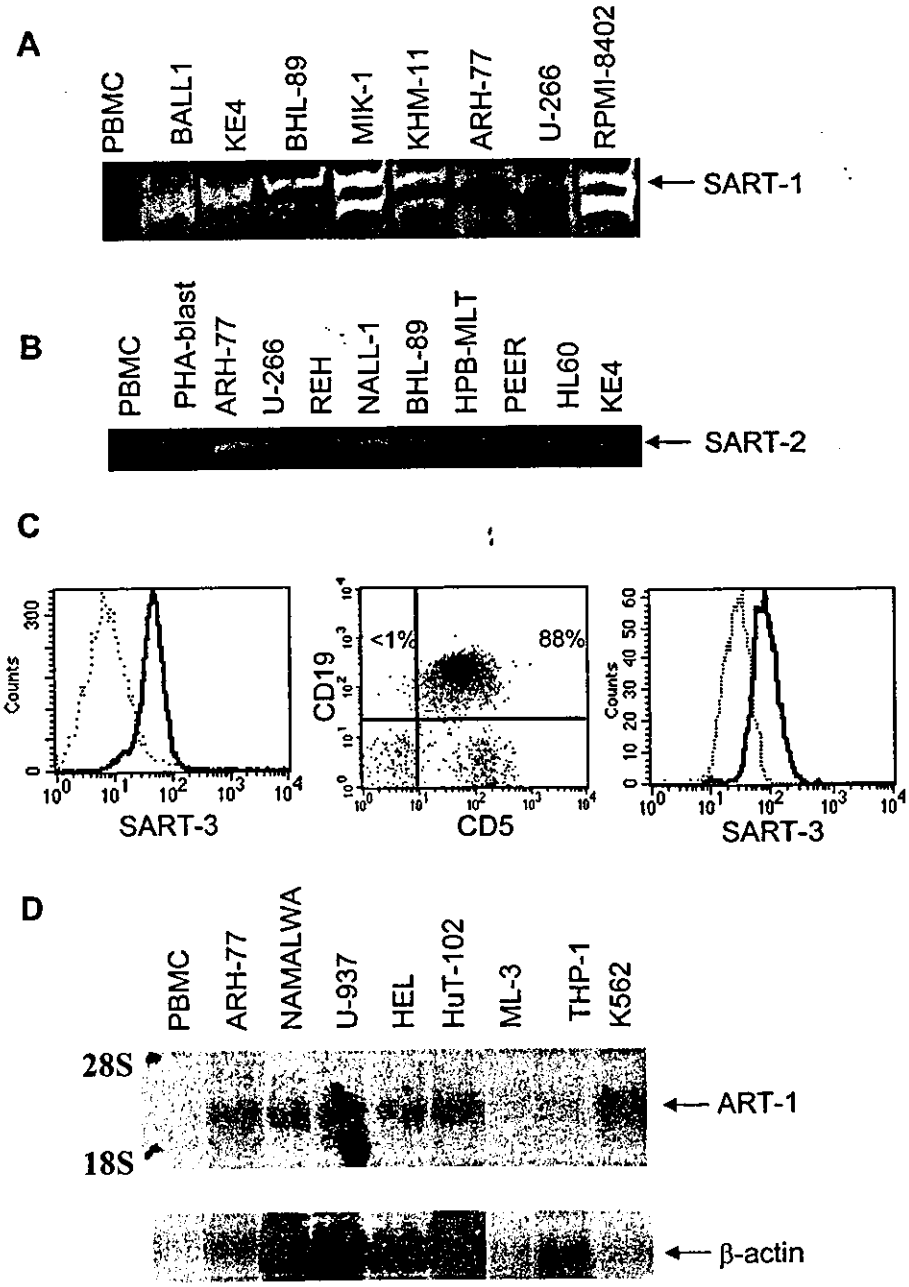

FIGURE 1. Expression of epithelial cancer-related antigens in hematologic malignancies. (A, B) The expression of SART-1 and SART-2 proteins in a panel of cell lines was examined by Western blot analysis using polyclonal anti-SART-1 and anti-SART-2 antibody. KE4 esophageal cancer cells, from which these antigens were cloned, and PBMCs were used as positive and negative controls, respectively. The cell lines used were B-cell leukemia and lymphoma cell lines (BALL-1, BHL-89, NALL-1, and REH), myeloma cell lines (MIK-1, KHM-11, ARH-77, and U-266), T-cell leukemia cell lines (RPMI 8402, PEER), an ATL cell line (HPB-MLT), and a myeloid leukemia cell line (HL60). (C) The expression of SART-3 in the BHL-89 cell line and in CLL cells from patient 1 was examined by flow cytometry. The BHL-89 cell line was stained with anti-SART-3 monoclonal antibody, followed by FITC-conjugated anti-mouse IgG secondary antibody (left). The PBMCs from patient 1 were stained with phycoerythrin (PE)-conjugated anti-CD5 monoclonal antibody and FITC-conjugated anti-CD19 monoclonal antibody (middle). The number represents a positive percentage in the subset. The CD19^{+} leukemia cells were examined for their expression of the cytoplasmic SART-3 with anti-SART-3 monoclonal antibody (right). The dotted line indicates an isotype-matched control. (D) The mRNA expression of the *ART-1* gene was examined by Northern blot analysis. The samples used were PBMCs from a healthy donor, a myeloma cell line (ARH-77), a Burkitt cell line (NAMALWA), AML cell lines (U-937, ML-3, and THP-1), a T-cell lymphoma cell line (HuT-102), an erythroleukemia cell line (HEL), and a CML cell line (K562). The mRNA expression of the β-*actin* gene was used as a control.

by flow cytometry with anti-SART-3 monoclonal antibody. The SART-3 was expressed in all malignant cell lines tested. Representative results of a histogram of the tumor cells are shown in Figure 1C. The BHL-89 cell line was positively stained with anti-SART-3 monoclonal antibody. A histogram of chronic lymphocytic leukemia (CLL) cells in the PBMCs of one patient (patient 1) is also shown in Figure 1C. In this histogram, most of the $CD19^+$ cells were $CD5^+$, implying that these $CD19^+$ cells were CLL cells expressing the SART-3 antigen in the cytoplasm.

Expression of the *ART-1* gene was investigated by Northern blot analysis (see Fig. 1D). This gene was expressed in all hematologic malignant cells tested and also in PBMCs. In most of the cell lines, however, the expression of the *ART-1* gene was higher than that in PBMCs. The expression of the $p56^{lck}$ in hematologic malignant cell lines was examined by two different methods. The Western blot analysis revealed that the $p56^{lck}$ protein was expressed in the HPB-MLT leukemia and ARH-77 myeloma cell lines, PBMCs, and PHA-blastoid T cells (Fig. 2A). The mRNA expression of the *p56lck* gene was further examined by the RT-PCR method, since the two different promoters, type I and type II, are preferentially used in malignant cells and normal PBMCs, respectively.[11] Therefore, we tested which promoters were used in hematologic malignancies, and representative results are shown in Figure 2B. The HPB-MLT, the PEER, and the ML-2 used both promoters, whereas the BALL-1, the BHL-89, and the RAJI used only the type I promoter. Overall results are summarized in Tables 1 and 2. These results indicate that the majority of hematologic malignant cell lines expressed the epithelial cancer-related antigens tested.

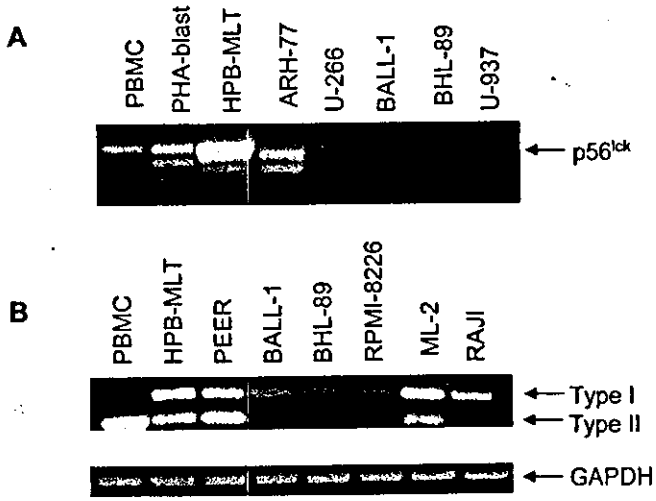

FIGURE 2. Expression of $p56^{lck}$ in hematologic malignancies. (A) The expression of the $p56^{lck}$ protein in a panel of cell lines was examined by Western blot analysis using anti-$p56^{lck}$ monoclonal antibody. Both PBMC and PHA-activated T-cell blasts were used as positive controls. The cell lines used were an ATL cell line (HPB-MLT), myeloma cell lines (ARH-77 and U-266), B-cell leukemia and lymphoma cell lines (BALL-1 and BHL-89), and a monocytic leukemia cell line (U-937). (B) The expression of two different promotor transcripts of the *lck* gene in a panel of cell lines was examined by RT-PCR. The cell lines newly used were T-cell acute lymphocytic leukemia (PEER), multiple myeloma (RPMI-8226), acute myelogenous leukemia (ML-2), and Burkitt lymphoma (RAJI). The expression of GAPDH was used a control.

CTL Induction From Patients With Hematologic Malignancies

PBMCs of 10 HLA-$A24^+$ patients with hematologic malignancies (2 with CLL, 2 with lymphoma, 1 with B-ALL, 1 with myeloma, 1 with myelodysplastic syndrome [MDS], 2 with ATL, and 1 with T-ALL) were tested for their ability to react to each of 10 different peptides, all of which were reported to induce peptide-specific and tumor-reactive CTLs from PBMCs of HLA-$A24^+$ epithelial cancer patients, and thus are under clinical trials as vaccines for epithelial cancer patients at the Kurume University Hospital.[12,13] PBMCs were stimulated in vitro with each of the indicated peptides, and their peptide-specific IFNγ production was determined. The results of the quadruplicate assays are shown in Figure 3. CTL precursors reactive to at least one peptide were observed in PBMCs from 8 of 10 patients. The mean positive number was 2.4 peptides per patient (range, 0–8 peptides). CTL precursors reactive to an EBV-derived peptide, as a positive control peptide, were detectable in 6 of 10 patients, whereas those to the HIV peptide, as a negative control, were undetectable in any patients. The profile of peptides to which CTL precursors showed their reactivity entirely varied among patients. PBMCs from all five patients with B-cell lymphoma/leukemia (patients 1 through 5) possessed no CTL precursors reactive to the Lck peptides. The IFNγ production by the peptide-stimulated PBMCs from two CLL patients (patients 1 and 2) in response to peptide-pulsed C1R-A24 cells was inhibited by the addition of anti-HLA class I, anti-CD8, and anti-HLA-A24 monoclonal antibody, but not of anti-CD14 monoclonal antibody (Fig. 4). Similarly, the IFNγ production by peptide-stimulated PBMCs from a MM patient (patient 6) in response to an HLA-$A24^+$ MM cell line was inhibited by the addition of anti-HLA class I, anti-CD8, and anti-HLA-A24 monoclonal antibody (see Fig. 4).

Peptide-stimulated PBMCs from seven patients (patients 1, 2, 4, 6, 7, 8, and 9) were further cultured for more than 2 weeks with IL-2 alone, and their cytotoxicity against both hematologic malignant cell lines and freshly prepared tumor cells, only when they were available, were examined by a standard 6-hour ^{51}Cr-release assay. These peptide-stimulated PBMCs exhibited significant levels of cytotoxicity against HLA-A24+ hematologic malignant cells and HLA-$A24^+$ fresh tumor cells (patients 1 and 2, auto-CLL cells; patient 6, auto-plasma cells; patient 9, allo-ATL cells) but not against HLA-