Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method

Tetsuya Nakatsura[1,2], Satoru Senju[1], Masaaki Ito[2], Yasuharu Nishimura[1] and Kyogo Itoh[2]

[1] Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan
[2] Department of Immunology, Kurume University School of Medicine, Kurume, Fukuoka, Japan

Among a number of human tumor antigens identified using the serological analysis of recombinant cDNA expression libraries (SEREX), only MAGE-1, tyrosinase, and NY-ESO-1 have been reported to be immunogenic tumor antigens that have the potential to elicit both humoral and cellular immunity. In this study, we determined whether our SEREX-defined pancreatic cancer antigens could be recognized by CTL, and report that one SEREX-defined antigen, coactosin-like protein (CLP), encoded cellular epitopes recognized by HLA-A2-restricted and tumor-reactive CTL. Three CLP peptides at positions 15–24, 57–65, and 104–113 possessed the ability to induce HLA-A2-restricted and tumor-reactive CTL from the PBMC of cancer patients. Subsequently, humoral responses to these peptides were investigated. IgG antibodies specific to the CLP 15–24, 57–65, and 104–113 peptides were detected in sera from 12, 0, and 12 of 12 cancer patients tested, and were also found in 5, 0, and 0 of 9 healthy donors, respectively. IgE antibodies specific to these peptides were also detected in sera from certain cancer patients and healthy donors. Since peptide-specific IgE was detected, type-I allergy to these peptides was tested. Unexpectedly the CLP 57–65 peptide, to which IgE was found in only 2 healthy donors, but not the other two peptides, was found to elicit an immediate-type hypersensitivity in all 10 healthy volunteers tested. These results indicate that identical antigenic peptides can be recognized by both cellular and humoral immune systems to a tumor-associated antigen. The CLP 15–24 and 104–113 peptides might be appropriate vaccine candidates for peptide-based immunotherapy of HLA-A2$^+$ cancer patients.

Key words: SEREX / Coactosin-like protein / Cancer antigen / CTL / Antibody

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1 Introduction

The recent development of molecular technology for analyzing cellular and humoral immune reactivity to cancer cells at the gene level has led to the identification and characterization of a large number of human tumor antigens recognized by CD8$^+$ T cells and antibodies. Many genes encoding tumor antigens and peptides that are recognized by CTL have been identified by cDNA expression cloning methods [1–6], thereby introducing the possibility of a peptide-based cancer immunotherapy. Post-vaccination PBMC became to show CTL activity against tumor cells in the clinical studies of peptide-based cancer immunotherapy, but these immunotherapies have rarely resulted in tumor regression [7, 8]. The failure to obtain tumor regression could be in part due to that immunogenicity of these tumor antigenic peptides was not strong enough to induce adaptive immunity against tumor cells. From this point of view, peptides with the ability to induce both CTL responses and humoral immunity could be better than those with the ability to induce either one. Over 1,500 types of tumor antigens have been identified using the serological analysis of recombinant cDNA expression libraries (SEREX) method [9–11]. However, only three (MAGE-1, tyrosinase, and NY-ESO-1) have been reported to have the ability to elicit both cellular and humoral immune responses to tumor cells [12–15]; CTL responses to the

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The last two authors contributed equally to this work.

Abbreviations: SEREX: Serological analysis of recombinant cDNA expression libraries **TIL:** Tumor-infiltrating lymphocyte **CLP:** Coactosin-like protein

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majority of SEREX-defined tumor antigens remain to be elucidated. Furthermore, there is no report on antigenic epitopes recognized by both cellular and humoral immune system to tumor-associated antigens.

We previously reported 18 SEREX-defined pancreatic cancer antigens [16]. This study has extended these studies and investigated whether five SEREX-defined pancreatic cancer antigens could be recognized by tumor-reactive CTL. We also provide evidence that one of the antigens is able to encode the identical epitopes recognized by both the cellular and the humoral immune system.

2 Results

2.1 Recognition of coactosin-like protein-derived peptides by an HLA-A2-restricted and tumor-reactive CTL line

Five genes coded for SEREX-defined pancreatic cancer antigens were considered from the perspective of whether or not their products could be recognized by an HLA-A2-restricted and tumor-reactive OK-CTL line by co-transfection of these cDNA and *HLA-A*0207* cDNA into COS-7 cells. This cell line was established from the tumor-infiltrating lymphocytes (TIL) of a patient (OK) with colon cancer, and responds to tumor cell lines in an HLA-A2-restricted manner, as reported previously [4, 6]. The OK-CTL line produced a significant level of IFN-γ in response to the COS-7 cells transfected with both *KM-PA-4* and *HLA-A*0207* cDNA (Fig. 1A). Maximum production of IFN-γ was observed when 100 ng *KM-PA-4* cDNA was transfected. In contrast, the OK-CTL line failed to produce significant levels of IFN-γ in response to COS-7 cells transfected with both *KM-PA-4* cDNA and an irrelevant *HLA-A*2402* cDNA, or with one of four kinds of cDNA (*KM-PA-5*, *KM-PA-14*, *KM-PA-15*, and *KM-PA-18*) together with *HLA-A*0207* cDNA. In our previous study [16], the KM-PA-4 was found to encode coactosin-like protein (CLP) consisting of 142 amino acids, as shown. These results indicate that KM-PA-4/CLP-derived antigens could be recognized by HLA-A2-restricted and tumor-reactive CTL. To determine the antigenic peptides recognized by the OK-CTL, eight different CLP-derived peptides with HLA-A2 binding motifs were prepared, and these peptides were then loaded onto T2 cells at a concentration of 10 μM; samples were tested for the ability to induce IFN-γ production by the OK-CTL (Fig. 1B). Three peptides (CLP 15–24, CLP 57–65, and CLP 104–113) had the ability to induce IFN-γ production by the OK-CTL.

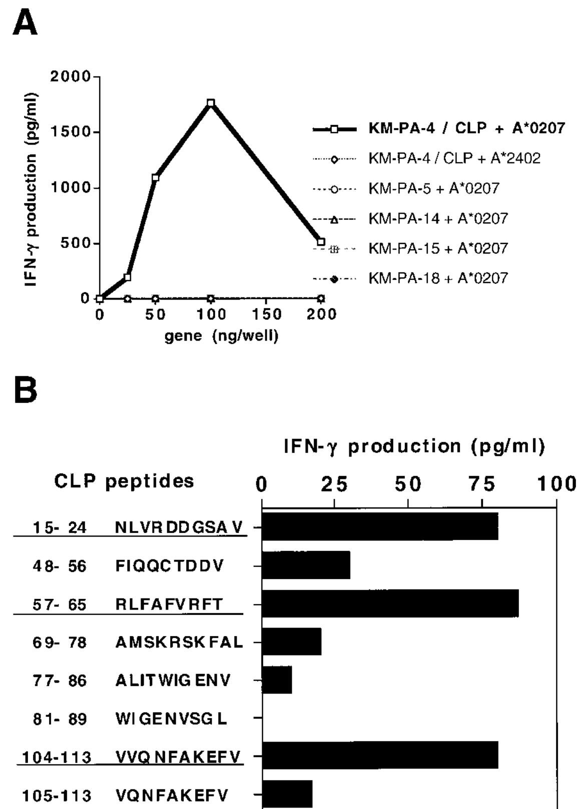

Fig. 1. Recognition of CLP-derived peptides by HLA-A2-restricted and tumor-reactive CTL. One of the highly reproducible results in a triplicate assay is indicated. (A) Different amounts of five SEREX-defined cDNA clones and 100 ng *HLA-A*0207* or *-A*2402* cDNA were co-transfected into COS-7 cells, followed by a test of the capacity to stimulate IFN-γ production by the HLA-A2-restricted and tumor-reactive OK-CTL. The experimental values, with the background production of IFN-γ release by the CTL in response to untransfected COS-7 cells (less than 100 pg/ml) subtracted, are shown. (B) Eight CLP-derived peptides with HLA-A2 binding motifs were loaded onto T2 cells at a concentration of 10 μM, and were tested for their ability to induce IFN-γ production by the OK-CTL. The experimental values, with the background production of IFN-γ (less than 30 pg/ml) by the CTL in response to T2 cells pre-loaded with the irrelevant HIV peptide subtracted, are shown. Three peptides, which were judged to be positive for this assay, are underlined.

2.2 Induction of tumor-reactive CTL by CLP peptides

We next attempted to generate tumor-reactive CTL from the PBMC of HLA-A2⁺ patients with pancreatic or colon cancer, or of HLA-A2⁺ healthy donors. The PBMC were stimulated with either the CLP 15–24, CLP 57–65, or CLP 104–113 peptide. These *in vitro*-sensitized PBMC

were tested for their cytotoxicity against various kinds of tumor cell lines in a 6-h ^{51}Cr-release assay (Fig. 2). The mRNA expression of the *CLP* gene in these cell lines used as target cells was confirmed by both reverse transcription (RT)-PCR and Northern blot analysis (data not shown). The CLP 15–24 peptide-sensitized PBMC from three of five cancer patients and two of six healthy donors showed significant levels of CTL activity against HLA-A2$^+$ Panc-1 and YPK-3 cells, but not against HLA-A2$^-$ PaCa-2 cells, HLA-A2$^+$ PHA-blasts or an HLA-A2$^+$ EBV-B cell line (Fig. 2). This was also the case with the CLP 57–65 peptide-sensitized PBMC in three of eight cancer patients, and in two of nine healthy donors (Fig. 2). Similarly, the CLP 104–113 peptide-sensitized PBMC from three of five cancer patients and one of seven healthy donors showed HLA-A2 restricted and tumor-reactive CTL activity (Fig. 2). The summary is shown in Table 1. In all cases, the percentage of CD8$^+$ T cells in peptide-stimulated PBMC was more than 80% (data not shown). These PBMC produced IFN-γ in response to T2 cells pre-loaded with a corresponding peptide in a dose-dependent manner (data not shown), and their IFN-γ production in response to HLA-A2$^+$ Panc-1 was inhibited by the addition of anti-HLA-class I, anti-CD8 or anti-HLA-A2 mAb (data not shown). These results indicate that these cytotoxicities were mediated by the peptide-specific and HLA-A2-restricted CD8$^+$ CTL.

2.3 Detection and quantification of serum IgG and IgE antibodies reactive to the CLP-derived peptides

We determined whether IgG specific to the whole CLP antigen could be detected in sera from pancreatic cancer patients and healthy donors using the SEREX method. IgG antibodies against the CLP antigen were found in the sera from 9 of 10 pancreatic cancer patients, and from 3 of 10 healthy donors (data not shown). We then examined if the three CLP peptides identified above could be recognized by the serum antibodies from cancer patients and healthy donors. ELISA was used to quantify levels of serum IgG and IgE specific to the CLP-derived peptides. Serum samples were judged as positive for peptide-specific antibody when the absorbance (unit) values changed in relation to the dilution of the serum samples; representative results are shown in

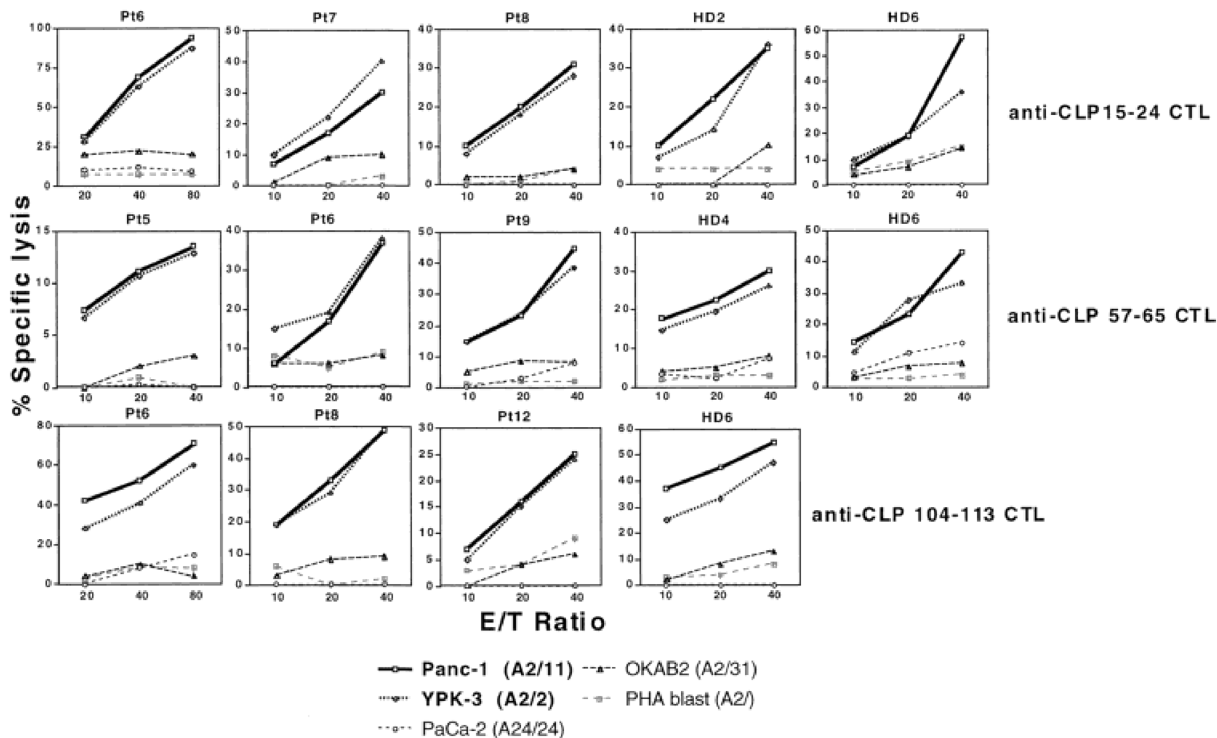

Fig. 2. Cytotoxic activity of PBMC stimulated with the CLP peptides. PBMC from patients with cancer and from healthy donors, listed in Table 1, were stimulated with the indicated peptides, followed by a test of the cytotoxicity against HLA-A2$^+$ Panc-1 and YPK-3, HLA-A2$^-$ PaCa-2 pancreatic cancer cell lines, HLA-A2$^+$ PHA-blasts, and the HLA-A2$^+$ EBV-B cell line, OKAB2, by a 6-h ^{51}Cr-release assay. Values represent the mean of triplicate assays. One of two highly reproducible results is indicated.

Table 1. Serum levels of IgG and IgE specific to the CLP peptides and induction of peptide-specific CTL from PBMC of HLA-A2$^+$ donors

	CLP 15-24			CLP 57-65			CLP 104-113		
HLA-A2$^+$ Donor	CTL induction	IgG	IgE	CTL induction	IgG	IgE	CTL induction	IgG	IgE
Pancreatic Cancer									
Pt1	NT[c]	3.7[d]	0.1	-	<0.1	<0.1	NT	1.0	<0.1
Pt2[a]	NT	4.5	<0.1	NT	<0.1	<0.1	NT	2.3	<0.1
Pt3	NT	6.0	0.4	-	<0.1	<0.1	NT	1.7	0.1
Pt4	NT	2.3	<0.1	-	<0.1	<0.1	NT	1.6	<0.1
Pt5	NT	3.8	1.9	+	<0.1	<0.1	NT	1.5	1.5
Pt6	+[e]	5.2	0.3	+	<0.1	<0.1	+	2.6	<0.1
Pt7	+	3.7	0.4	NT	<0.1	<0.1	-	1.0	1.0
Pt8	+	4.5	<0.1	NT	<0.1	<0.1	+	2.0	<0.1
Colon Cancer									
Pt9 (OK)[b]	-	4.5	0.1	+	<0.1	<0.1	-	2.0	<0.1
Pt10	NT	4.4	0.5	-	<0.1	<0.1	NT	1.9	0.4
Pt11	NT	5.2	<0.1	NT	<0.1	<0.1	NT	2.1	<0.1
Pt12	-	4.3	0.5	-	<0.1	<0.1	+	1.8	1.4
Healthy Donor									
HD1	-	0.3	0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD2	+	1.9	0.4	-	<0.1	0.7	-	<0.1	0.1
HD3	NT	1.8	<0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD4	NT	2.0	0.2	+	<0.1	<0.1	NT	<0.1	1.1
HD5	NT	1.6	<0.1	-	<0.1	<0.1	NT	<0.1	<0.1
HD6	+	<0.1	<0.1	+	<0.1	<0.1	+	<0.1	<0.1
HD7	-	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD8	-	<0.1	0.2	-	<0.1	<0.1	-	<0.1	<0.1
HD9	-	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	<0.1

a) The *KM-PA-4/CLP* was identified by the SEREX method using serum from this patient [16].

b) The OK-CTLs were established from this patient [4, 6].

c) NT: not tested.

d) The results of IgG and IgE antibodies binding to either the CLP 15-24, CLP 57-65 or CLP 104-113 peptide are shown by 10X absorbance values (10X OD unit) in ELISA. The experimental values, from which the background absorbance (10X OD unit) without peptide was subtracted, are shown. Serum samples were diluted at x100 for IgG and at x2 for IgE, respectively. Values judged as positive for peptide-specific antibody in ELISA are underlined.

e) The symbol represents successful induction of peptide-specific and HLA-A2$^+$ tumor-reactive CTL. The results are shown in Fig. 2.

Fig. 3A and B. A decrease in the absorbance unit of IgG against both the CLP 15–24 and CLP 104–113 peptides, but not against the CLP 57–65 peptide, was observed in serum dilutions in all 4 patients tested (Fig. 3A). As with IgE, sera from Pt5 and Pt7, and HD4 were evaluated as positive for IgE to both the CLP 15–24 and CLP 104–113 peptides, but not against the CLP 57–65 peptide (Fig. 3B). In the case of HD2, serum dilution resulted in a decrease in the absorbance of IgE against all three peptides (Fig. 3B). A competitive binding inhibition assay was performed to confirm the peptide specificity of IgG or IgE detected in this ELISA system. Detection of IgG or IgE antibody was inhibited by adding a corresponding peptide to the sample serum in a dose-dependent manner, but not by adding an irrelevant peptide (data not shown). These results indicate that an excess of a free peptide in serum samples showed a peptide-specific competition against the coating peptide, validating the peptide specificity of this ELISA system.

IgG and IgE specific to the CLP peptides were measured in sera of 12 HLA-A2$^+$ patients with cancer (Pt1–12) (8 pancreatic cancer and 4 colon cancer) and 9 HLA-A2$^+$ healthy donors (HD1–9), summarized in Table 1. IgG specific to the CLP 15–24 peptide was detected in the sera of all 12 cancer patients, and was also found in 5 of 9 healthy donors, while IgE specific to this peptide was detected in the sera of 8 of 12 cancer patients and of 4 of

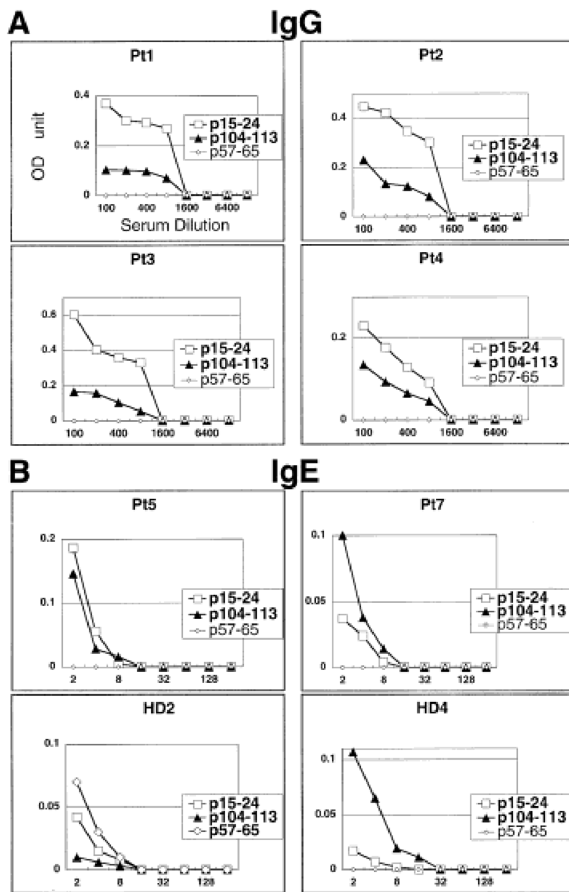

Fig. 3. Detection and quantification of serum IgG and IgE antibodies specific to the CLP peptides. One representative result of two highly reproducible experiments is shown. Peptide-reactive IgG and IgE in serially diluted serum samples from cancer patients and healthy donors were examined in (A) and (B), respectively.

9 healthy donors. IgG and IgE specific to the CLP 104–113 peptide were detected in the sera of 12 and 5 of 12 cancer patients, and in the sera of 0 and 2 of 9 healthy donors, respectively. In contrast, no IgG reactive to the CLP 57–65 peptide was detected in any of the donors tested. IgE reactive to the CLP 57–65 peptide was detected in only 1 healthy donor (HD2). Similar results were observed in HLA-A2^{-} cancer patients and healthy donors for antibodies reactive to the CLP 15–24 and CLP 104–113 peptides, but IgG specific to the CLP 104–113 peptide was detected in the sera of 4 of 10 HLA-A2^{-} healthy donors (data not shown). There was no significant association between the presence of the CLP 15–24 or CLP 104–113 peptide-specific IgG antibody in sera and successful induction of CTL from PBMC *in vitro* (p=0.546 and 0.222, respectively, evaluated by the χ^2 test). There was no inverse correlation between the existence of IgE antibody and successful CTL induction. CLP 15–24 or CLP 104–113 peptide-specific CTL were induced from PBMC, regardless of the detection of serum IgE specific to these peptides.

2.4 Type-I allergic reaction to the CLP 57–65 peptide

Type-I allergic reactions to some CTL epitopes were observed in the skin tests of cancer patients in the phase I clinical trial of peptide-based immunotherapy, and those patients who showed type-I allergic reaction against the peptide were not vaccinated with the peptide, because of the risk of systemic anaphylaxis (Gouhara et al., unpublished results). Because serum IgE reactive to the CLP peptides were detected in certain patients and healthy donors (Table 1), we investigated whether these CLP-derived CTL epitope peptides could elicit type-I allergic reactions in five HLA-A2^{+} healthy and five HLA-A2^{-} healthy volunteers. The CLP 57–65 peptide elicited the type-I allergy in all ten healthy volunteers tested, whereas the other two peptides failed to induce the type-I reaction in any donors.

The CLP 57–65 peptide and the analogue peptides in which individual amino acid residues were substituted by glycine (glycine scan) were prepared to gain a better understanding of the molecular basis of the type-I allergy, and the results of the skin test with the CLP 57–65 analogue peptides are listed in Table 2. The glycine substitution at positions 1 and 7 resulted in a decrease of the type-I allergic reaction, suggesting that position 1 (arginine) and position 7 (arginine) were important for the type-I allergic reaction to the CLP 57–65 peptide. On the other hand, substitution at position 6 resulted in an increase of the type-I allergic reaction in all cases tested. The results of IgE binding to analogues of the CLP 57–65 peptide are listed in Table 3. The glycine substitution at position 5, 6, or 9 in HD2 or at position 6, 7, and 9 in HD10 resulted in a complete or a moderate loss of IgE recognition, respectively. There was a discrepancy between important amino acid residues for the type-I allergic reaction and those for IgE antibody binding. These peptide analogues were also tested for the ability to stimulate the CLP 57–65 peptide-stimulated PBMC to produce IFN-γ. In contrast to the results of the type-I allergic reaction and antibody binding, recognition by peptide-specific CTL was lost in the majority of glycine-substituted analogue peptides (data not shown).

Table 2. Results of skin test with the CLP 57-65 analogue peptides [a)]

CLP-derived peptides		Donors [b)] HD2 (HLA-A2$^+$, IgE$^+$)	HD6 (HLA-A2$^+$, IgE$^-$)	HD10 (HLA-A2$^-$, IgE$^+$)	HD11 (HLA-A2$^-$, IgE$^-$)
		Area of redness (cm^2)			
57-65	RLFAFVRFT	100	100	100	100
R57G	GLFAFVRFT	36.0 [c)]	17.4	39.1	16.7
L58G	RGFAFVRFT	114	100	156	100
F59G	RFGAFVRFT	114	100	56.3	52.9
A60G	RLFGFVRFT	114	156	66.0	82.6
F61G	RLFAGVRFT	64.0	156	56.3	67.0
V62G	RLFAFGRFT	128	278	352	186
R63G	RLFAFVGFT	16.0	17.4	31.6	13.2
F64G	RLFAFVRGT	114	178	66.0	151
T65G	RLFAFVRFG	100	50.2	100	20.7

a) The results of the skin test with analogues of the CLP 57-65 peptide are shown as relative percentages compared with those with the wild-type peptide.

b) Skin test was tested in 10 healthy volunteers, and the results of 4 donors (HLA-A2$^+$ or -A2$^-$, positive or negative of serum IgE specific to the CLP 57-65 peptide) are shown in the table.

c) Values showing more than 50% reduction, compared with those with the wild-type peptide, are underlined.

3 Discussion

The SEREX-defined KM-PA-4 gene was highly expressed at the mRNA level in pancreatic cancer cell lines as compared with normal pancreatic tissues in our previous study [16]. The sequence of the *KM-PA-4* gene was identical to that of the *CLP* gene (derived from human placenta) already registered in the GenBank (accession no. L54057). CLP was referred to because CLP protein product (142 amino acids) showed a significant homology to coactosin [17], a filamentous (F)-actin-binding protein from *Dictyostelium discoideum*, with 33.3% identity in amino acid sequence [18]. The mRNA of CLP is 1824 bp long and is expressed predominantly in placenta, lung, liver, and kidney, but not in the heart, brain, skeletal muscle, and pancreas [18]. Smith-Magenis syndrome (SMS), which involves the clinical symptoms of mental retardation, neuro-behavioral abnormalities, sleep disturbances, short stature, minor craniofacial and skeletal anomalies, congenital heart defects, and renal anomalies, is caused by deletion of the short arm of chromosome 17 in band p11.2. *CLP* gene is mapped to the SMS common deletion region [18]. This SMS critical region overlaps with a breakpoint cluster region associated with primitive neuroectodermal tumors, suggesting that CLP plays a role in DNA rearrangements of somatic cells [18]. CLP has also been demonstrated to interact directly with 5-lipoxygenase (5LO), which plays a pivotal role in cellular leukotriene synthesis [19, 20]. 5LO appeared to compete with F-actin for the binding of CLP. Further studies are needed to clarify the biological functions of CLP.

It has been reported that the same immunodominant myelin basic protein peptides were important for antibody binding and Th cell recognition in multiple sclerosis patients [21]. However, there has been no report of antibodies against class I-associated CTL epitopic peptides. In this study, we provided evidence of IgG and IgE antibodies against CTL epitopic peptides. We suppose that IgG and IgE antibodies detected by ELISA were specific to the CLP peptides based on the following: (1) serial dilution of serum resulted in a proportional decrease in antibody binding to the peptides; (2) addition of free peptides inhibited the peptide-specific binding of antibodies in serum to coating peptides, as shown in the competitive binding inhibition assay; and (3) peptide binding was significantly influenced by several analogue peptides with only one amino acid substitution. These results validate the specificity of IgG and IgE against the CLP peptides. On the other hand, IgG and IgE antibodies specific to the CLP-derived peptides were detected in the sera of HLA-A2-negative donors in similar proportion to that of both HLA-A2$^+$ cancer patients and healthy donors (data not shown). Determination of class II-associated epitopes of the CLP antigen is critically important to fully understand T cell response to the CLP.

Table 3. Binding of IgE antibodies to the CLP 57-65 analogue peptides[a)]

CLP-derived peptides		Donor HD2	Donor HD10
		IgE bound to analogue peptides (OD unit) [b)]	
57-65	RLFAFVRFT	100 (0.067)	100 (0.113)
R57G	GLFAFVRFT	140 (0.094)	77.0 (0.087)
L58G	RGFAFVRFT	139 (0.093)	162 (0.183)
F59G	RFGAFVRFT	118 (0.079)	92.0 (0.104)
A60G	RLFGFVRFT	85.1 (0.057)	85.0 (0.096)
F61G	RLFAGVRFT	0[c)] (0)	85.0 (0.096)
V62G	RLFAFGRFT	0 (0)	15.9 (0.018)
R63G	RLFAFVGFT	85.1 (0.057)	10.6 (0.012)
F64G	RLFAFVRGT	97.0 (0.065)	61.9 (0.070)
T65G	RLFAFVRFG	0 (0)	29.2 (0.033)

a) The levels of IgE antibodies binding to analogues of the CLP 57-65 peptide are shown as relative percentages compared with those of the wild-type peptide.

b) Absorbance values (OD units) are shown in parentheses. The values were subtracted by the background absorbance (OD units) without peptides.

c) Values showing more than 50% reduction, compared with those bound to the wild-type peptide, are underlined.

Three patterns of existence of peptide-specific IgG antibodies were shown in the three CLP peptides. IgG antibodies against the CLP 15–24 peptide were detected in the sera from all of the cancer patients; these antibodies were also found in half of the healthy donors. Antibodies against the CLP 104–113 peptide were detected in the sera from all cancer patients, and they were found in none of the healthy donors. Antibodies against the CLP 57–65 peptide were not detected in the sera from any of the cancer patients or the healthy donors. In the present study, we found that CLP-derived peptide-specific and tumor-reactive CTL could be induced predominantly in cancer patients. In particular, CLP 104–113 peptide-specific and tumor-reactive CTL activity was induced in three of five cancer patients (60.0%), and in one of seven healthy donors (14.3%). Thus, the CLP 104–113 peptide may have the highest potential among the three peptides to induce tumor-specific cellular and humoral immunity in PBMC of cancer patients. On the other hand, we could not induce CTL against various peptides in some of the patients. We think that these variations may be partly due to the heterogeneity of tumor cells and T cell repertoires in each patient. There may be difference in precursor frequency of peptide-specific CTL in each individual. It is also possible that cellular immunity of some cancer patients may be depressed. The number of cancer patients who had CTL precursors reactive to EBV peptide was significantly lower than that of healthy donors (unpublished observation).

The type-I allergic reaction to the CLP 57–65 peptide was not restricted to HLA-A2^{+} donors. This type of reaction to some CTL epitopes was also observed in pre-vaccinal skin tests of cancer patients in the phase I clinical trial of peptide-based immunotherapy (Gouhara et al., unpublished results). Allergy is classically defined as an immunological reaction to a foreign antigen [22]. However, anaphylactic shock to a self peptide was recently described in mouse EAE [23]. Injection of a myelin proteolipid protein 139–151 peptide in mice after immunization with the same peptide was reported to cause anaphylactic shock. The type-I allergic reaction to the CLP 57–65 peptide was observed in all donors tested, whereas detectable levels of IgE were only found in sera from two donors (HD2 and 10). The CLP 57–65-specific IgE may be trapped on the surface of mast cells by a high-affinity FcεR-I, and only a small amount of IgE, at levels too low to detect, is present in the circulation.

There was a discrepancy between important amino acid residues for the type-I allergic reaction and for IgE antibody binding. This observation may, in part, explain why IgE reactive to the CLP 57–65 peptide was not detected in the majority of donors who exhibited type-I allergic reaction. Glycine substitution at position 6 (V62G) resulted in an increase of type-I allergic reaction. This could be due to the molecular mimicry between microbial non-self peptides and V62G analogue of the CLP 57–65. *Escherichia coli, Mycobacterium tuberculosis*

and many other microbes have sequences sharing a six-amino acid sequence identity with V62G. On the other hand, the type-I allergic reaction and IgE binding activity was lost when glycine substitution was introduced in relatively localized amino acid residues, whereas CTL recognition was lost in the majority of the glycine-substituted analogue peptides. Both positions 2 and 9 of the peptides are considered to be anchor residues, and residues at other positions are thought to be TCR contact residues. The loss of CTL recognition in analogue peptides carrying a glycine substitution at position 2 or 9 is most likely due to the failure of peptide binding to HLA-A2, because glycine does not have a side chain. The loss of CTL recognition in their analogue peptides might be due to low affinity or to the lack of binding of CTL to TCR, because positions 1, 3, 4, 5, 6, 7, and 8 of the CLP 57–65 peptide have side chains of considerable size.

CLP is a self antigen, and its mRNA is expressed in some normal tissues [16, 19], as well as being overexpressed in pancreatic cancer cell lines, compared to normal pancreatic tissues. CTL induced by stimulation with CLP-derived peptides showed cytotoxicity against cancer cell lines, but not against two kinds of proliferating T and B lymphoblasts (Fig. 2). These results suggest that vaccination with these peptides were not associated with adverse effects on normal cells and tissues. Some self antigens expressed in certain normal tissues prove to be good candidates for cancer immunotherapy. For example, immunizing patients with normal self peptides derived from melanosomal proteins has resulted in dramatic tumor regression with only occasional vitiligo in some patients who had been prescribed immunotherapy with melanoma epitopes together with administration of IL-2 [24]. Further study of immune responses of self-antigen-recognizing and tumor-reactive CTL to normal cells and tissues is required.

NY-ESO-1 protein is thought to be the most immunogenic antigen, since it has been reported to have the ability to elicit both cellular and humoral immune responses to tumor cells. However, there are no reports on tumor-associated antigenic epitopes recognized by both cellular and humoral immune systems. We provide evidence that CLP can encode identical epitopes recognized by both the cellular and humoral immune systems. Further study is needed to clarify whether these peptides have the ability to induce orchestrated anti-tumor immune responses of not only CTL, but also Th cells and antibodies.

In conclusion, we identified three CLP peptides that were capable of propagating HLA-A2-restricted and tumor-reactive CTL from PBMC. One of these peptides, CLP 57–65, elicits a type-I allergic reaction. The HLA-A2 allele is found in 23% of Black Africans, 53% of Chinese, 40% of Japanese, 49% of Northern Caucasians, and 38% of Southern Caucasians [25]. These results indicate that the CLP 15–24 and CLP 104–113 peptides could be appropriate candidates in use for specific immunotherapy for a large number of cancer patients.

4 Materials and methods

4.1 HLA-A2-restricted CTL line

An HLA-A2-restricted and tumor-reactive CTL line, OK-CTL, was used to investigate whether SEREX-defined genes could encode antigens recognized by CTL. This cell line was established from the TIL of a colon cancer patient OK (HLA-A*0207/*3101, -B46/51, -Cw1) by incubation in medium supplemented with IL-2 (100 U/ml) alone for more than 50 days, the details of which have been described elsewhere [4, 6].

4.2 Analysis for antigen recognition by CTL

Eighteen candidate genes encoding pancreatic cancer antigens were identified using serum from Pt2 (HLA-A*0210/*2402, -B*5201/*4006, -DRB1*1502/*09012, -DQB1*0601/03) with pancreatic cancer through SEREX screening of a cDNA library generated from a human pancreatic adenocarcinoma cell line, CFPAC-1 [16]. Among them, five types of full-length cDNA clones encoding either KM-PA-4/CLP, KM-PA-5/HALPHA55, KM-PA-14/CGI55 protein, KM-PA-15/GIF, or KM-PA-18/hsp105, which were packaged in the EcoRI- and XhoI-digested pBluescript vectors, were inserted into the expression vector pCMV-SPORT-2 (Life Technologies, Rockville, MD). The cDNA of *HLA-A*0207* or *-A*2402* genes was obtained by RT-PCR and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). cDNA (0–200 ng) of the of each of the SEREX-defined genes and 100 ng *HLA-A*0207* or *-A*2402* cDNA were suspended in 50 μl Opti-MEM (Life Technologies), mixed with Lipofectamine™ reagent (Life Technologies), and samples were cultured at room temperature for 30 min. A 50-μl aliquot of the mixture was then added to the COS-7 cells (5×10^3), which were then incubated for 6 h. Thereafter, the COS-7 cells were cultured for 2 days in RPMI 1640 medium supplemented with 10% FCS, followed by the addition of CTL (5×10^4 cells/well). After an 18-h incubation, 100 μl of the culture supernatant was collected and assayed in triplicate for IFN-γ production by ELISA.

4.3 Peptides

Eight different CLP-derived peptide candidates with the potential to bind to the HLA-A2 molecules [26, 27] were synthesized (Fig. 1B). The peptides and their analogues with a

single amino acid substitution to glycine were synthesized by Fmoc/PyBOP. These peptides were purchased from Sawady Laboratory (Tokyo, Japan), and their purity, estimated by HPLC, was >70%. For the additional studies, three peptides, including the CLP 15–24, 57–65, and 104–113 peptides, with a purity of >95%, were prepared. As a negative control, an HLA-A2-binding HIV-derived peptide (SLYNTYATL) with a purity of 90–98%, kindly provided by Dr. Kanaoka (Sumitomo Pharmaceutical, Osaka, Japan), was used.

4.4 Cancer cell lines

The cancer cell lines used in this study and their HLA-A alleles, shown in parentheses, were as follows: Panc-1 (HLA-A*0201/1101) pancreatic adenocarcinoma, YPK-3 (HLA-A*0201) pancreatic adenocarcinoma, PaCa-2 (HLA-A*2402) pancreatic adenocarcinoma, and RERF-LC-MS (HLA-A*1101) lung adenocarcinoma. HLA-A2$^+$ EBV-transformed B cell lines and HLA-A2$^+$ PHA-activated T lymphocytes were used as control cells.

4.5 *In vitro* sensitization of PBMC with the peptides

PBMC were isolated from 20 ml of heparinized blood of HLA-A2$^+$ cancer patient donors and healthy donors by Ficoll-Conray density gradient centrifugation, as reported previously [5]. Informed consent was obtained from all donors. HLA class I typing was performed on blood lymphocytes using the classical serological method [5]. A simple method was used to generate peptide-specific CTL from PBMC (Hida et al., unpublished results). In brief, PBMC (1×10^5 cells/well) were incubated with 10 μM peptide in 200 μl culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI 1640 medium, 45% AIM-V® medium (Gibco-BRL), 10% FCS, 100 U/ml of recombinant human IL-2, and 0.1 μM MEM nonessential amino acid solution (Gibco-BRL). Half of the medium was removed and replaced with fresh medium containing the corresponding peptide (20 μM) every 3 days for up to 12 days.

4.6 Assays for tumor antigen-reactive T cell responses

Peptide-stimulated PBMC were further expanded in the presence of feeder cells pre-pulsed with the indicated peptide for 21–28 days to obtain a relatively large number of cells. Thereafter, they were tested for their responses against various targets by a 6-h ^{51}Cr-release assay [5]. The surface phenotypes of the CTL were examined by direct immunofluorescence staining with FITC-conjugated anti-CD3, -CD4, or -CD8 mAb (Nichirei, Tokyo, Japan) [6]. To determine both effector cells and MHC restriction, 20 μg/ml anti-HLA-class I (W6/32, IgG2a), anti-HLA-A2 (BB7.2, IgG2b), anti-CD8 (Nu-Ts/c, IgG2a), anti-HLA-DR (H-DR-1, IgG2a), and anti-CD4 (Nu-Th/i, IgG1) mAb were added at the beginning of the cultures. Anti-CD13 (MCS-2, IgG2a) and anti-CD14 (JML-H14, IgG1) mAb were used as isotype-matched negative controls [6].

4.7 Skin test

Each peptide was dissolved in DMSO at 10 mg/ml, aseptically aliquoted, and stored at –80°C. Stock solutions were diluted with saline just before use. A sterility test was performed according to the method described in Section B-484 of the Japanese Pharmacopoeia 13th edn. Peptide solution (50 μl, 0.2 mg/ml) was injected intradermally into healthy volunteers and, 15 min later, flare and induration were inspected. Informed consent was obtained from all participants.

4.8 Detection of serum IgG reactive to the entire CLP antigen

SEREX-positive clones of CLP were subcloned and retested for serum reactivity, according to the following method. To determine the reactivity of other allogeneic sera samples (1:100 dilution) obtained from ten healthy blood donors (50–63 years old, mean 56.7 years, four men and six women) and ten patients with pancreatic ductal adenocarcinoma (43–79 years old, mean 62.1 years, four men and six women) against SEREX-positive clones, plates containing an equal number of sero-positive and sero–negative control clones were similarly processed. The immunoscreening method has been described previously [16].

4.9 Measurement of serum IgG and IgE reactive to the peptides

An ELISA was used to quantify serum levels of IgG and IgE reactive to the CLP-derived peptides. Immobilization of the peptides at their C termini to a 96-well Nunc Covalink flat plate (Fisher Scientific, Pittsburgh, PA) using disuccinimidyl suberate (Pierce, Rockford, IL) was performed according to the manufacturer's instructions. The plate of immobilized peptides (10 μg/well) was blocked with Block Ace (Yukijirushi, Tokyo, Japan), and washed with 0.05% Tween 20-PBS (PBST); 100 μl/well of either serum or plasma samples diluted with 0.05% Tween 20-Block Ace was then added to the plate. The plate was washed with PBST after 2-h incubation at 37°C, and further incubated for 2 h at 37°C with 1:1,000-diluted rabbit anti-human IgG antibodies (Dako, Glostrup, Denmark). The plate was washed nine times, and 100 μl of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase-dextran polymer (EnVision, Dako) was added to each well, and the plate was incubated at room temperature for 40 min. After washing, 100 μl/well of tetramethyl-benzidine substrate solution (KPL, Guildford, GB) was added, and the reaction was stopped by the addi-

tion of 1 M H_3PO_4. Absorbance was measured at 450 and 630 nm. As with the IgG-measurement procedure, levels of IgE were measured using 1:1,000-diluted rabbit anti-human IgE (Dako). Analogues of the CLP 57–65 peptide, in which the indicated positions were substituted by glycine, were used to determine amino acid positions crucial for the type-I allergic reaction, antibody binding, and CTL recognition.

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References

1 **Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T. and Rosenberg, S. A.,** Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 6458–6462.

2 **Boon, T. and van der Bruggen, P.,** Human tumor antigens recognized by T lymphocytes. *J. Exp. Med.* 1996. **183:** 725–729.

3 **Shichijo, S., Nakao, M., Imai, Y., Takasu, H., Kawamoto, M., Niiya, F., Yang, D., Toh, Y., Yamana, H. and Itoh, , K.,** A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. *J. Exp. Med.* 1998. **187:** 277–288.

4 **Ito, M., Shichijo, S., Miyagi, Y., Kobayashi, T., Tsuda, N., Yamada, A., Saito, N. and Itoh, K.,** Identification of SART3-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients with different HLA-A2 subtypes. *Int. J. Cancer* 2000. **88:** 633–639.

5 **Harashima, N., Tanaka, K., Sasatomi, T., Shimizu, K., Miyagi, Y., Yamada, A. Tamura, M., Yamana, H., Ito, K. and Shichijo, S.,** Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. *Eur. J. Immunol.* 2001. **31**: 323–332.

6 **Ito, M., Shichijo, S., Tsuda, N., Ochi, M., Harashima, N., Saito, N. and Ito, K.,** Molecular basis of T cell-mediated recognition of pancreatic cancer cells. *Cancer Res.* 2001. **61:** 2038–2046.

7 **Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Prakhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H. and White, D. E.,** Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med.* 1998. **4:** 321–327.

8 **Wang, F., Bade, E., Kuniyoshi, C., Spears, L., Jeffery, G., Marty, V., Groshen, S. and Weber, J.,** Phase I trial of a MART-1 peptide vaccine with incomplete Freund's adjuvant for resected high-risk melanoma. Clin. Cancer *Res.* 1999. **59:** 4056–4060.

9 **Sahin, U., Tureci, O., Schmitt, H., Cochlovius, B., Johannes, T., Schmits, R., Stenner, F., Luo, G., Schobert, I. and Pfreundschuh, M.,** Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc. Natl. Acad. Sci. USA* 1995. **92:** 11810–11813.

10 **Stockert, E., Jager, E., Chen, Y. T., Scanlan, M. J., Gout, I., Karbach, J., Arand, M., Knuth, A. and Old, L. J.,** A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J. Exp. Med.* 1998. **187:** 1349–1354.

11 **Scanlan, M. J., Chen, Y. T., Williamson, B., Gure, A. O., Stockert, E., Gordan, J. D., Tureci, O., Sahin, U., Pfreundschuh, M. and Old, L. J.,** Characterization of human colon cancer antigens recognized by autologous antibodies. *Int. J. Cancer* 1998. **76:** 652–658.

12 **van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A. and Boon, T.,** A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991. **254:** 1643–1647.

13 **Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P. and Boon, T.,** The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 1993. **178:** 489–495.

14 **Jager, E., Chen, Y. T., Drijfhout, J. W., Karbach, J., Ringhoffer, M., Jager, D., Arand, M., Wada, H., Noguchi, Y., Stockert, E., Old, L. and J., Knuth, A.,** Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J. Exp. Med.* 1998. **187:** 265–270.

15 **Jager, E., Gnjatic, S., Nagata, Y., Stockert, E., Jager, D., Karbach, J., Neumann, A., Rieckenberg, J., Chen, Y. T., Ritter, G., Hoffman, E., Arand, M., Old, L. J. and Knuth, A.,** Induction of primary NY-ESO-1 immunity: $CD8^+$ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1^+ cancers. *Proc. Natl. Acad. Sci. USA* 2000. **97:** 12198–12203.

16 **Nakatsura, T., Senju, S., Yamada, K., Jotsuka, T., Ogawa, M. and Nishimura, Y.,** Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. *Biochem. Biophys. Res. Commun.* 2001. **256:** 75–80.

17 **de Hostos, E.L., Bradtke, B., Lottspeich, F. and Gerisch, G.,** Coactosin, a 17 kDa F-actin binding protein from Dictyostelium discoideum. Cell Motil. *Cytoskeleton* 1993. **26:** 181–191.

18 **Chen, K.S., Manian, P., Koeuth, T., Potocki, L., Zhao, Q., Chinault, C., Lee, C.C. and Lupski, J.R.,** Homologous recombination of a flanking repeat gene cluster is a mechanism for a common contiguous gene deletion syndrome. *Nat. genet.* 1997. **17:** 154–163.

19 **Provost, P., Samuelsson, B. and Radmark, O.,** Interaction of 5-lipoxygenese with cellular proteins. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 1881–1885.

20 **Provost, P., Doucet, J., Hammarberg, T., Gerisch, G., Samuelsson, B. and Radmark, O.,** 5-Lipoxygenase interacts with coactosin-like protein. J. *Biol. Chem.* 2001. **276:** 16520–16527.

21 **Ota, K., Matsui, M., Milford, E. L., Mackin, G.A., Weiner, H. L. and Hafler, D. A.,** T cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* 1990. **346:** 183.

22 **Galli, S. J. and Lantz, C. S.,** Allergy. In **Paul, W.E.** (Ed.) *Fundamental immunology.* Lippincott-Raven Press, Philadelphia 1999, pp 1127–1174.

23 **Pedotti, R., Mitchell, D., Wedemeyer, J., Karpuj, M., Chabas, D., Hattab, E. M., Tsai, M., Galli, S. J. and Steinman, L.,** An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat. Immunol.* 2001. **2:** 216–222.

24 **Kawakami, Y.,** Identification of human melanoma antigens recognized by tumor infiltrating T lymphocytes and their use for immunotherapy. In **Kikuchi, K., van den Eynde, B. J. and Sato, N.** (Eds.) *Recent advances of human tumor immunology and*

immunotherapy. Japan Scientific Societies Press, Tokyo 1999, pp 179–189.

25 **Imanishi, I., Akazawa, T., Kimura, A., Tokunaga, K. and Gojobori, T.,** Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In **Tsuji, K., Aizawa, M. and Sasazuki, T.** (Eds.) *Proceedings of the 11th International Histocompatibility Workshop and Conference*. Oxford University Press, Oxford 1992, pp 1065–1220.

26 **Parker, K. C., Bednarek, M. A. and Coligan, J. E.,** Scheme for ranking potential HLA-A2 peptides based on independent binding of individual peptide side-chains. *J. Immunol.* 1994. **152:** 163–175.

27 **Rammensee, H. G., Friede, T. and Stevanoviic, S.,** MHC ligands and peptide motifs: first listing. *Immunogenetics* 1995. **41**: 178–228.

Correspondence: Kyogo Itoh, Department of Immunology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan
Fax: +81-942-31-7699
e-mail: kyogo@med.kurume-u.ac.jp

or Yasuharu Nishimura, Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Honjo 2-2-1, Kumamoto 860-0811, Japan
Fax: +81-96-373-5314
e-mail: mxnishim@gpo.kumamoto-u.ac.jp