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Non-mutated tumor-rejection antigen peptides elicit type-I allergy in the majority of healthy individuals

Key words:

autoantibody; autoantigen; immediate-hypersensitivity; cytotoxic T-lymphocytes; peptides

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Abstract: IgE-mediated type-I allergy is generally considered to be a hypersensitivity reaction to foreign antigens, and it is believed that self-antigens do not evoke this type of allergy. We report here, for the first time, that non-mutated self-antigen peptides identified as tumor-rejection antigen peptides recognized by HLA class I-restricted and tumor-specific cytotoxic T lymphocytes (CTLs) elicited a type-I allergy in the majority of healthy individuals. Peptide-specific IgE was detectable in sera from certain cases, although the levels did not always correlate with those of type-I allergy. Repeated vaccinations of nonallergic peptides derived from the same antigens possessing allergic peptides resulted in the suppression of both allergic peptide-specific IgE responses and type-I allergy, providing evidence for a new approach to the development of peptide-based desensitization.

It is known that immune effector mechanisms can lead to severe tissue damage if turned against the host; in 1900, Ehrlich referred to this mechanism as a “horror autotoxicus” (1, 2). Indeed, it has been shown that some self-peptides elicit anaphylactic shock in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis (3). Cloning and sequencing of cDNAs encoding allergic molecules has revealed that these molecules are non-mutated self-antigens, and now there is accumulating evidence that IgE-mediated reactions against self-antigens could play a role in severe atopic diseases (4–12). Using similar molecular techniques, we have defined many tumor-rejection antigens from cDNA libraries of epithelial cancer cells over the past decade (13–20). These antigens recognized by host cytotoxic T lymphocytes (CTLs) were mostly non-mutated self-antigens that play various roles in cellular proliferation and thereby are preferentially expressed in both malignant cells and proliferating normal cells. Non-mutated self-antigens recognized by IgE of patients with atopic dermatitis and those recognized by the CTLs of cancer patients share many characteristics, including their preferential intra-

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cellular location in the nucleus. Indeed, two of the non-mutated self-antigens, squamous cell antigen recognised by T cells (SART) 1 and cyclophilin B (CypB), identified by our group as tumor-rejection antigens (14, 15), have also been identified by other investigators as antigens involved in the auto-allergic process of atopic dermatitis (12, 21).

In the course of developing a peptide-based specific immunotherapy for cancer patients during the prevaccination skin test that aids in the avoidance of anaphylactic shock, we discovered the type-I allergy to non-mutated self-antigen. In the present study, we demonstrated evidence for type-I allergy to non-mutated self-peptides in healthy individuals. Our investigations into the mechanisms underlying this phenomenon are presented here, as well as a new approach to the development of peptide-based desensitization.

Materials and methods

Subjects

Forty-one healthy volunteers ranging in age from 19 to 54 years (mean age 29.2 years) were entered in this study as subjects for skin tests and as serum donors. None of the healthy volunteers had any clinical history of any allergic diseases, and all of their serum IgE levels were normal (less than 250 IU/mL by RIST). Sera from cancer patients attending phase-I clinical trials of peptide cancer vaccines at Kurume University Hospital were also used for this study. Protocols of the clinical trials were reviewed and approved by the Kurume University Review Board and the independent Ethical Committee (protocol numbers 9905, 9910 and 9928). All patients were required to comprehend and sign an informed consent form before entry into the clinical trial. Healthy volunteers (research staff in our laboratory and students from our medical school) also provided their informed consent before entry into this study.

Peptides and skin test

The peptides used in this study were prepared by Multiple Peptide Systems (San Diego, CA) under the conditions of Good Manufacturing Practice. The following peptides, all of which can bind the HLA-A24 molecule and have the ability to induce CTLs (13, 15, 19, 20) and, all of which have been used as cancer vaccines in clinical trials, were employed: CypB-derived peptides at positions 84–92 (CypB₈₄, KFHRVIKDF) and 91–99 (CypB₉₁, DFMIQGGDF), adenocarcinoma antigen recognised by T cells 4 (ART4)-derived peptides at positions 13–20 (ART4₁₃, AFLRHAAL) and 75–84 (ART4₇₅, DYPSSLATDI), SART2-derived peptides at positions 161–169 (SART2₁₆₁, AYDFLY-NYL) and 899–907 (SART2₈₉₉, SYTRLFLIL), and SART3-derived

peptides at positions 109–118 (SART3₁₀₉, VYDYNCHVDL) and 315–323 (SART3₃₁₅, AYIDFEMKI). Modified peptides of CypB₈₄ and ART4₁₃, in which one of the amino acids was substituted for glycine (CypB₈₄–1G~9G and ART4₁₃–1G~8G), were supplied by Sumitomo Pharmaceuticals (Osaka, Japan) and Sawady Technology (Tokyo, Japan), respectively, and were used for the fine-epitope analyses. Peptides were >90% pure, as assessed by reverse-phase high-pressure liquid chromatography analyses. Each peptide was dissolved in dimethyl sulfoxide (DMSO), stored at –80°C, and diluted with saline immediately before use. Ten micrograms of peptide (50 µL in volume) were intradermally injected, and allergic skin reactions were assessed 15 min after the injection. Saline containing the equivalent concentration of DMSO was used as a control.

Quantification of peptide-specific IgE and IgG

Peptide-specific IgE and IgG levels in sera were measured by ELISA. Immobilization of the peptides to a 96-well Nunc Covalink flat plate (Fisher Scientific, Pittsburgh, PA) with disuccinimidylsuberate (PIERCE, Rockford, IL) was performed according to the manufacturer's instructions. The peptide (20 µg/well)-immobilized plates were blocked with Block Ace (Yukijirushi, Hokkaido, Japan) and washed with 0.05% Tween 20-PBS (PBST). One hundred µL/well of serum or plasma samples diluted with 0.05% Tween 20-Block ace were added to the plates. After a 2-h incubation at 37°C, the plates were washed with PBST and further incubated for 2 h at 37°C with 1:1000-diluted rabbit antihuman IgE (ε-chain-specific), antihuman IgG (γ-chain-specific) (DAKO, Glostrup, Denmark), or antihuman IgG subclass-specific antibody (Zymed Laboratories, South San Francisco, CA). The plates were washed nine times and then 100 µL of 1:100-diluted goat antirabbit Ig-conjugated horseradish peroxidase-dextran polymer (EnVision; DAKO) were added to each well, and the plates incubated at room temperature for 40 min. After washing, 100 µL/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) were added, and the reaction was stopped by the addition of 1 M phosphoric acid. To estimate peptide-specific IgE levels, the optical density (OD) values of each sample were compared with those of serially diluted standard samples, and these values are shown as OD units/mL. Peptide-specific IgG levels were similarly estimated. For the standard wells, 1:100-diluted antihuman IgE (Southern Biotechnology Associates, Birmingham, AL) or antihuman IgG (Leinco, Ballwin, MO) mAbs were immobilized instead of the peptides. Cut-off values of the peptide-specific IgE or IgG were determined using HIV 9-mer peptide as solid phase antigen. Values of the HIV-specific IgE or IgG of the 41 healthy volunteers were less than 0.01 or 0.02 OD units, respectively. Therefore these values were used as cut-off values. A clinical laboratory (SRL,

Summary of skin tests and serum levels of IgE and IgG specific to vaccine peptides

Healthy donors						CypB84			CypB91		
ID no.	Gender	HLA-A	HLA-DR	Total IgE	Aspergillus fumigatus IgE	ST	IgE ^a	IgG ^b	ST	IgE	IgG
1	M	24/26	1/6	51	0.11	++	0.048	–	–	–	–
2	M	24/33	2/9	240	0.34	+	0.155	–	–	0.077	–
3	M	24	6/9	99	0.09	++	–	–	–	–	0.099
4	M	2402/0201	2	31	0.12	++	–	–	–	–	–
5	M	24	4/9	190	0.09	–	–	0.39	–	–	0.27
6	M	11/31	2/8	73	0.08	++	0.215	–	–	–	–
7	M	24/26	2	19	0.08	++	–	–	–	–	–
8	M	24	1/4	33	0.12	+	–	–	–	–	–
9	F	0201/0206	4/9	220	0.19	+	–	–	–	–	–
10	M	24/2	4/9	230	0.31	+	–	–	–	–	–
11	M	2	6/12	18	0.07	++	–	–	–	–	–
12	M	31/33	2/4	7.1	0.07	++	–	–	–	–	–
13	M	0201/0207	8/9	190	0.07	+	0.053	–	–	0.069	–
14	M	0201/24	2/8	62	0.07	+	0.04	–	–	–	–
15	F	11/24	2	82	0.06	++	–	0.044	–	–	0.045
16	F	24/33	1/6	170	0.06	+	0.138	–	–	0.173	–
17	M	2409/2610	2	240	0.07	+	–	–	–	–	–
18	M	11/2409	2/6	16	0.04	++	–	NT	–	–	NT
19	M	2409/3319	2/9	200	0.15	+	0.027	NT	–	–	NT
20	M	2/2610	2/4	235	0.06	+	0.039	–	–	–	–
21	M	2409	4/11	32	0.05	++	–	NT	–	0.038	NT
22	F	11/24	1	120	0.05	+++	–	–	–	–	–
23	F	2/31	2/12	56	0.04	+	–	–	–	–	–
24	F	2409	4/8	220	0.05	+++	–	–	–	–	–
25	M	24/31	1/9	150	0.06	++	–	–	–	0.064	–
26	F	24/26	4	46	0.05	+	0.094	–	–	–	–
27	F	2/11	8/9	87	0.04	+	–	–	–	–	–
28	F	31	4	23	0.05	+	–	–	–	–	0.061
29	M	26/33	4/8	150	0.06	+++	–	NT	–	–	NT
30	F	24	4/12	120	0.05	++	0.092	–	–	0.053	–
31	F	24/33	4/6	240	0.05	+	–	–	–	–	–
32	M	24	NT	35	0.03	+	0.036	0.777	–	–	1.021
33	M	24	NT	130	0.16	++	–	–	–	–	–
34	M	24/31	4/8	120	0.05	+	–	–	–	–	–
35	F	24/26	2	120	0.04	++	–	–	–	–	–
36	F	2/24	2/4	81	0.04	+	–	–	–	–	–
37	F	11/24	NT	56	0.04	–	–	0.089	–	–	–
38	F	2	NT	110	0.06	+	0.026	–	–	–	–
39	F	11/24	NT	100	0.05	+	–	–	–	–	–
40	F	2	NT	82	0.04	+	–	–	–	–	–
41	M	26/33	NT	15	0.11	+	–	–	–	–	–
Positive rate						39/41	12/41	4/37	0/41	6/41	5/37

ST, skin test. Definition of the results of skin test is as follows: – = longest diameter (D) of flare <10 mm; + = 10 mm ≤ D ≤ 30 mm, ++ = 30 mm ≤ D < 50 mm, +++ = D ≥ 50 mm

NT, not tested

^aOD unit. A value of greater than 0.010 was considered to be positive^bOD unit. A value of greater than 0.020 was considered to be positive

Table 1

ID no.	ART4 13			ART4 75			SART4 161			SART2 899		
	ST	IgE	IgG	ST	IgE	IgG	ST	IgE	IgG	ST	IgE	IgG
1	+	0.047	-	-	-	-	-	-	-	-	-	-
2	++	-	-	-	-	-	-	NT	NT	++	NT	NT
3	++	-	-	-	-	0.162	-	-	-	+	-	-
4	+	-	-	-	-	-	-	-	-	+	-	-
5	-	-	-	-	-	0.118	-	-	-	++	-	0.095
6	++	-	-	-	-	-	-	-	-	-	-	-
7	++	-	-	-	-	-	-	-	-	+	-	0.024
8	+	-	-	-	-	-	-	-	-	-	-	-
9	+	-	-	-	-	-	-	-	-	+	-	-
10	+	-	-	-	-	-	-	-	-	-	-	-
11	++	0.112	-	-	-	-	-	-	-	-	-	-
12	++	-	-	-	-	-	-	-	-	-	-	-
13	+	-	-	-	-	-	-	-	-	-	-	-
14	NT	-	-	NT	-	-	-	-	-	NT	-	-
15	+	-	0.063	-	-	0.037	-	-	-	-	-	-
16	+	-	-	-	-	-	-	-	-	-	-	-
17	++	-	-	-	-	-	-	-	-	-	-	-
18	+	-	NT	-	-	NT	-	-	-	+	-	-
19	+	-	NT	-	-	NT	-	-	-	-	-	0.423
20	+	-	-	+	-	-	-	-	-	-	-	0.036
21	++	-	NT	-	-	NT	-	-	0.44	-	-	0.316
22	+	-	-	-	-	-	-	-	0.196	-	-	0.364
23	+	-	-	-	-	-	-	NT	-	-	NT	-
24	+	-	-	-	-	-	-	-	-	+	-	-
25	+	0.016	-	-	NT	-	-	-	-	-	-	-
26	+	-	-	-	-	-	-	-	-	-	-	-
27	+	-	-	-	-	-	-	-	0.117	-	-	-
28	+	-	0.053	-	-	0.052	-	-	-	-	-	-
29	++	-	NT	-	-	NT	-	-	-	-	-	-
30	+	-	-	-	-	-	-	0.024	-	-	-	-
31	+	-	-	-	-	-	-	-	-	-	-	-
32	+	0.044	-	-	-	0.395	-	-	-	-	-	0.354
33	++	-	-	-	-	-	-	-	-	-	-	-
34	+	-	-	-	-	0.054	-	-	-	+	-	-
35	+	-	-	-	-	-	-	-	-	-	-	-
36	+	-	-	-	-	-	-	-	-	+	-	-
37	-	-	-	-	-	-	-	-	-	-	-	-
38	+	-	-	-	-	-	-	-	-	-	-	-
39	+	-	-	-	-	-	-	-	-	-	-	-
40	+	0.014	-	-	-	-	-	-	-	-	-	-
41	++	-	-	-	-	-	-	-	-	-	-	-
Positive rate	38/40	5/41	2/37	1/40	0/40	6/37	0/41	1/39	3/40	10/40	0/39	7/40

Table 1. Continued

ID no.	SART3 109			SART3 315		
	ST	IgE	IgG	ST	IgE	IgG
1	-	-	-	-	-	-
2	++	0.133	-	-	-	-
3	-	-	0.375	-	-	-
4	-	-	-	-	-	-
5	-	-	0.247	-	-	0.458
6	-	-	-	-	-	-
7	+	-	-	+	-	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	+	0.021	-	-	-	-
11	-	-	-	-	0.04	-
12	-	-	-	-	-	-
13	-	0.065	-	-	0.025	-
14	-	0.034	-	NT	-	-
15	-	0.078	0.118	-	-	0.025
16	-	0.157	0.025	+	0.115	-
17	-	-	-	++	-	-
18	-	0.031	NT	+	-	NT
19	-	-	NT	+	-	NT
20	-	-	0.072	+	-	-
21	-	0.026	NT	-	0.021	NT
22	-	-	-	-	-	-
23	+	-	-	-	-	-
24	-	-	-	-	-	-
25	-	0.024	-	-	0.023	-
26	-	-	-	-	-	-
27	-	0.023	-	+	-	-
28	-	-	0.066	-	0.014	0.081
29	-	-	NT	++	-	NT
30	-	-	-	+++	-	-
31	-	-	-	+	-	-
32	+	0.071	0.0865	++	-	-
33	-	-	-	++	-	-
34	-	-	0.159	++	-	0.028
35	+	-	-	++	-	-
36	+	-	-	++	-	-
37	-	-	-	+	-	-
38	-	-	-	-	-	-
39	-	-	-	+	-	-
40	-	-	-	+	-	-
41	-	-	-	-	-	-
Positive rate	7/41	11/41	8/37	18/40	6/41	4/37

Table 1. Continued

Tokyo, Japan), measured the total IgE and Aspergillus allergen-specific IgE levels of the samples, including the standard samples. Direct comparison of the values of OD units in our system and LC units in MAST is not easy as the two assay systems are completely different. However, we can provide sample data of Aspergillus allergen-specific IgE levels in an atopic patient measured using the two assay systems. In this case, 1.73 LC in the MAST was comparable with 2.35 OD units in our system. Single-epitope-specific IgE might be several-fold lower than these values, since the Aspergillus allergen contained multiple epitopes.

Vaccination protocols

HLA-A24⁺ patients with advanced cancer were enrolled into this study. The peptides used were as follows: CypB₉₁ for lung cancer patients, ART4₇₅ for ovarian cancer patients and SART3₁₀₉ for colorectal cancer patients. No patient had received any treatment, steroids or other immunosuppressive drugs from 4 weeks prior to the study until study completion. If the skin test proved negative, 1 mL peptide solution (1 mg/mL) was emulsified with an equal volume of incomplete Freund's adjuvant (Montanide ISA-51; Seppic, Paris, France) and injected subcutaneously. It was intended that all patients would receive at least 3 vaccinations at 2-week intervals; when patients requested then, additional vaccinations were given for a total of 3–17 vaccinations per patient.

Results

Type-I allergy to self-peptides in healthy volunteers

Two cyclophilin B (CypB)-derived 9-mer peptides, CypB₈₄ and CypB₉₁, have been identified as tumor-rejection antigen peptides that are recognized by HLA-A2402-restricted CTLs (15). Their potential for use in a cancer vaccine was studied in a phase-I clinical trial. In the trial, the CypB₈₄ peptide, but not the CypB₉₁ peptide, was found to elicit immediate hypersensitivity skin reactions at pre-vaccination skin tests in 8 of 10 prevaccinated patients with lung cancer (Gohara et al. unpublished results). The other three peptides (ART4₁₃, SART2₈₉₉, SART3₃₁₅) among 6 peptides similarly elicited allergic skin reactions in prevaccinated patients during prevaccination skin tests in phase I clinical studies of a cancer vaccine (22). All of the 8 peptides shown in Table 1 were derived from non-mutated self-antigens, and had the ability to induce HLA-A24-restricted and tumor-specific CTL activity from the PBMCs of cancer patients (13, 15, 19, 20).

To improve understanding of the scientific basis of this unex-

pected phenomenon, we investigated whether immediate-type skin reactions to these 8 peptides would be observed in healthy volunteers ($n = 41$; 19–54 years old; mean age 29.2 years). The CypB₈₄, ART4₁₃, SART2₈₉₉, SART3₁₀₉, and SART3₃₁₅ peptides elicited immediate-type skin reactions in 39 of 41 (95%), 38/40 (95%), 10/40 (25%), 7/41 (17%), and 18/40 (45%) healthy individuals, respectively. In contrast, the CypB₉₁, ART4₇₅, and SART2₁₆₁ peptides did not elicit such a response. A wheal-and-flare allergic reaction developed within one minute after the intradermal injection of peptide; in most cases, the reaction peaked at 5–20 min, then gradually diminished and had completely disappeared by 60–90 min (Fig. 1). These findings indicate that such peptide-elicited skin reactions constitute a typical type-I allergy. No systemic anaphylactic reactions or late phase responses, such as Arthus reaction or delayed type hypersensitivity, were observed in any of the patients. A sum-

mary of the skin-test results is provided in Table 1. No correlation was found between these allergic skin reactions and HLA-A or -DR types, or between the reactions and the gender of the subjects (Table 1).

Peptide-specific IgE and IgG in healthy individuals

Serum levels of peptide-specific IgE and IgG were measured to facilitate a better understanding of the molecular basis of allergic skin reactions. Representative results of measurement by the peptide-specific enzyme-linked immunosorbent assay (ELISA) are shown in Fig. 2A. A linear inverse correlation was observed between the optical density (OD) values of CypB₈₄- or ART4₁₃-specific ELISA and serum dilutions. The specificity of the CypB₈₄- or ART4₁₃-specific ELISA was confirmed by inhibition tests with soluble peptides.

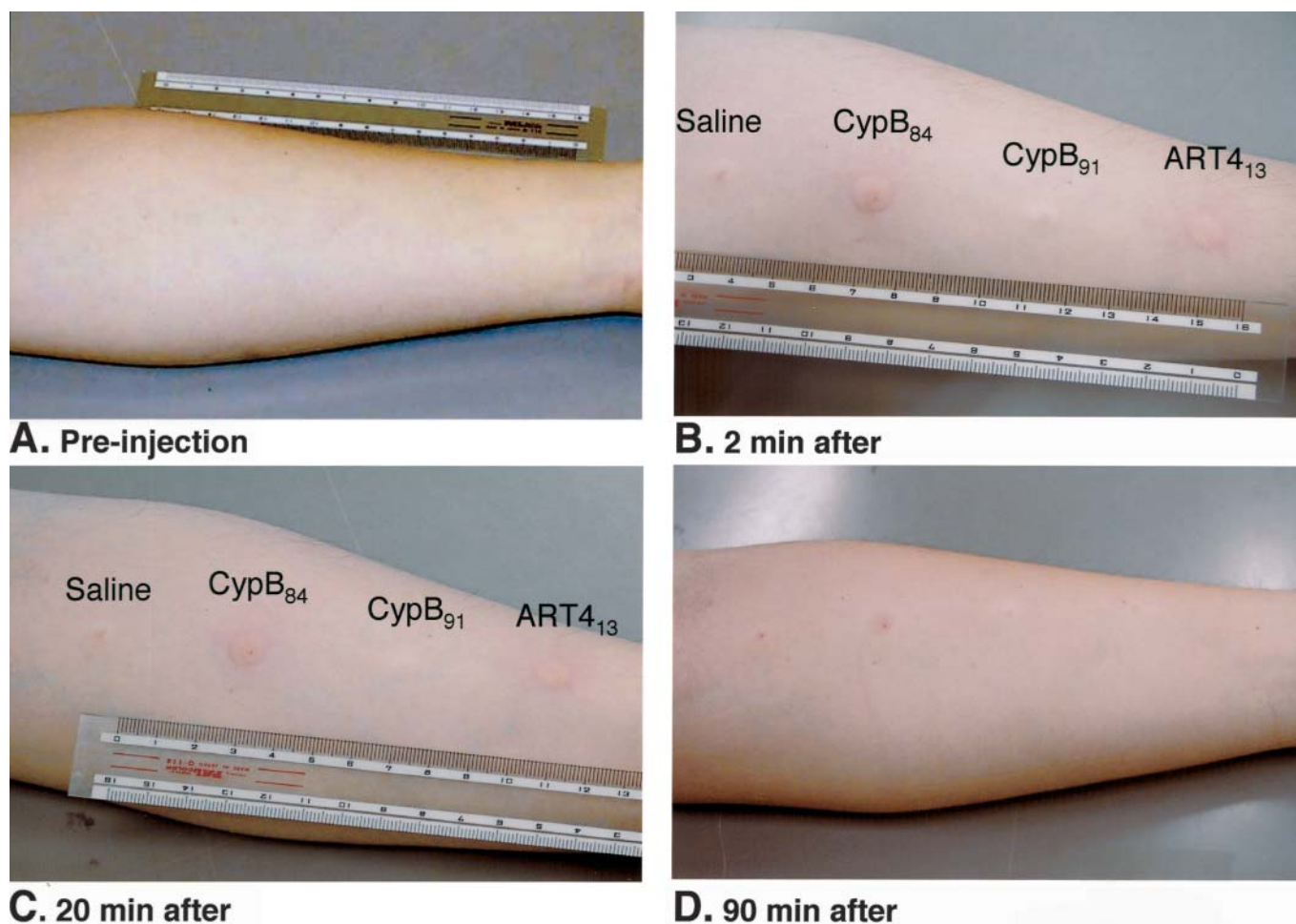


Fig. 1. Representative photographs of the skin test. Ten micrograms of CypB₈₄, CypB₉₁, ART4₁₃ peptide, or saline containing equivalent concentration of DMSO as a negative control (volume 50 μ L), were intradermally injected into a healthy individual, and allergic skin reactions were assessed at various time points (in min) after the injection.

Each peptide-specific ELISA was inhibited by the addition of a corresponding peptide, but not by irrelevant peptides. Representative results are shown in Fig.2B. Overall results of tests for serum IgE or IgG specificity for each of the various peptides are summarized in Table 1. CypB₈₄- or CypB₉₁-specific IgE was detected in 12 or 6 of 41 cases, respectively, whereas the CypB₈₄- or CypB₉₁-specific IgG was detectable in 4 or 5 of 37 cases tested. Among 39 individuals showing skin reactions to CypB₈₄, sera from 12 cases had detectable levels of IgE to the corresponding peptide, though the degree of skin reactions did not correlate with serum IgE levels.

ART4₁₃- or ART4₇₅-specific IgE was detected in 5 of 41 and 0 of 40 cases, respectively, while ART4₁₃- or ART4₇₅-specific IgG was detectable in 2 and 6 of 37 cases tested. SART2₁₆₁- or SART2₈₉₉-specific IgE was detected in 1 and 0 of 39 cases, respectively, while the corresponding IgGs were detectable in 3 or 7 of 40 cases tested, respectively. SART3₁₀₉- or SART3₃₁₅-specific IgE was detected in 11 and 6 of 41 cases, while the corresponding IgGs were detectable in 8 and 4 of 37 cases tested, respectively. Collectively, there was no obvious correlation between the level of *in vivo* skin reaction and those of serum IgEs or IgGs specific to the corresponding peptide.

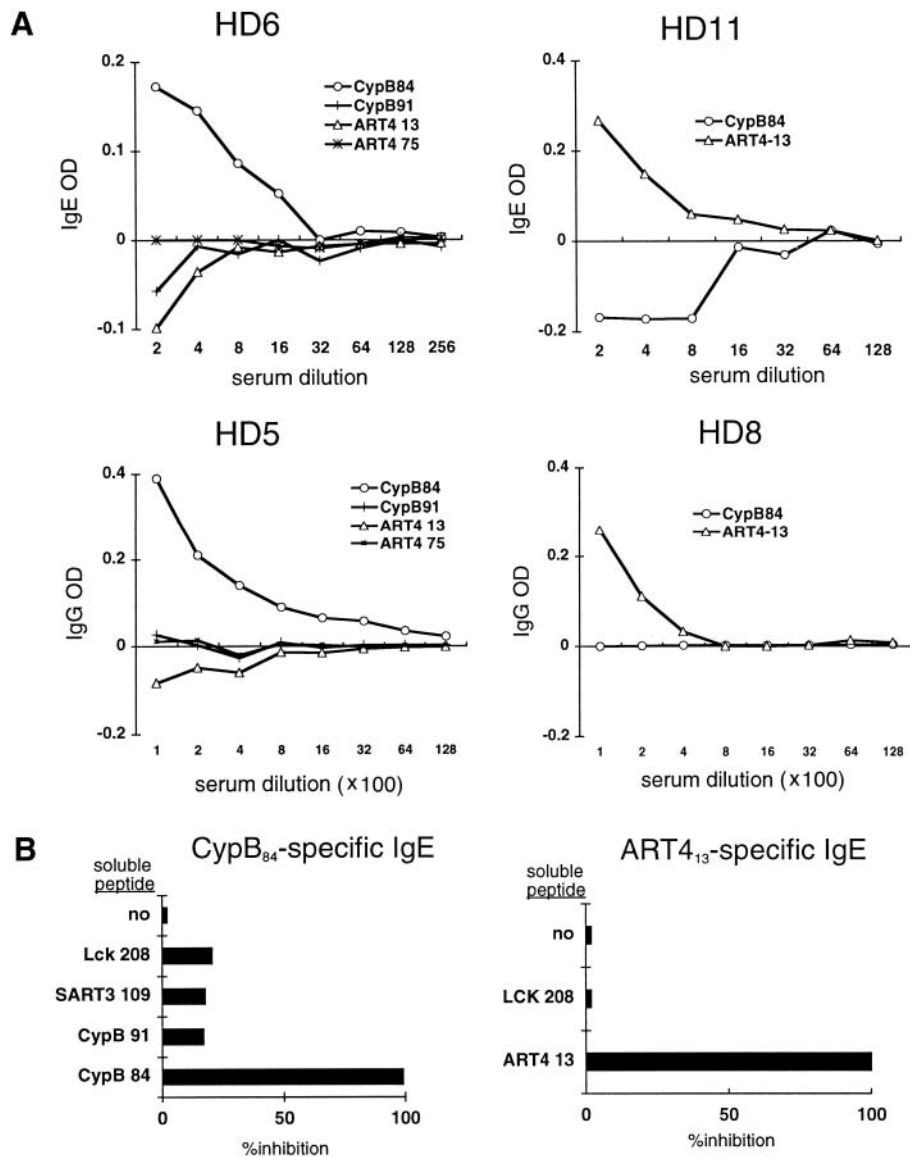


Fig. 2. Detection of serum level of peptide-specific IgE and IgG. (A) Representative results of CypB₈₄- or ART4₁₃-specific IgE or IgG ELISA are shown. (B) Inhibition of CypB₈₄- or ART4₁₃-specific IgE ELISA by soluble peptides. The soluble form of the indicated peptides (final concentration, 1 mg/mL) was mixed with the sample sera and was subjected to ELISA. Irrelevant peptides include Lck₂₀₈ peptides with the ability to induce CTLs¹⁸.

These findings were unexpected, as a good correlation is normally found between these two parameters when comparing responses to common allergens. It is of note that the sera of the two individuals (HD5, 37) that showed no skin reaction to the CypB₈₄ peptide had detectable levels of IgG but no detectable levels of IgE to the corresponding peptide.

The proportion of IgG subclass among total IgG and among peptide-specific IgG to the two CypB and ART4 peptides in the sera of 6 healthy volunteers (HD 3, 5, 15, 28, 32 and 37) were further investigated to improve understanding of the relative contributions of T helper type 1 and 2 cells in antibody production (2, 23). The proportions of T helper type 2-cell promoting antibodies (IgG4 and IgE) to the allergic peptides (CypB₈₄ and ART4₁₃) were not significantly different from those to the nonallergic peptides (CypB₉₁ and ART4₇₅). Namely, the dominant IgG subclass of the total IgG was IgG1 (59–79% of total IgG; mean: 69%) and IgG2 was subdominant (17–29%; mean: 21%). The relative contents of IgG3 and IgG4 were 8–3% and 6–2%, respectively. The subclass distributions of peptide-specific IgG toward each of the four peptides (CypB₈₄, CypB₉₁, ART4₁₃, ART4₇₅) were similar to those of the total IgG; i.e., the

relative contents of each subclass were 86–60%, 32–7%, 7–4% and 5–2%, respectively.

Amino acid substitution analyses

CypB₈₄ and ART4₁₃ peptides and their analog peptides, in which individual amino acid (aa) residues were substituted by glycine, were prepared to determine the aa residues important for the elicitation of skin reactions in 7 healthy volunteers (Table 2). Glycine substitution at positions 1, 2, 3, 4, 5, 6, 7, 8 or 9 of CypB₈₄ resulted in a significant reduction (≤ 0.25 of flare area ratio) of skin reactions in 1, 1, 2, 1, 0, 3, 1, 0 and 2 of 7 cases, respectively. These findings suggest that the critical aa residues largely vary among individuals. In contrast, glycine substitution at positions 2 and 4 of ART4₁₃ resulted in no skin reactions in all 4 cases tested, although substitutions at positions 3, 5, 6 and 8 also resulted in significant reduction in one of each case.

These peptides were used to determine the aa residues important for antibody recognition, and representative results are shown in Fig. 3. Amino acid residues at positions 1, 4, 7 and 9 of the CypB₈₄

Effects of glycine substitution on CypB₈₄ and ART4₁₃ elicited skin reaction

Peptides	Sequence	HD1	HD2	HD3	HD6	HD10	HD15	HD40	Suppressed cases FR ≤ 0.25
CypB ₈₄ original	KFHRVIKDF	600 ^a	1600	910	484	360	300	640	
CypB84–1G	GFHRVIKDF	49^b	1200	1600	300	300	195	224	1/7
CypB84–2G	KGHRVIKDF	750	1140	625	280	140	90	600	1/7
CypB84–3G	KFGRVIKDF	84	1140	750	300	320	75	400	2/7
CypB84–4G	KFHGVIKDF	49	825	500	150	225	104	182	1/7
CypB84–5G	KFHRGIKDF	660	2025	980	340	204	540	600	0/7
CypB84–6G	KFHRVGKDF	49	750	0	260	140	0	225	3/7
CypB84–7G	KFHRVIGDF	300	810	0	980	180	130	289	1/7
CypB84–8G	KFHRVIKGF	600	1350	1200	1050	320	506	234	0/7
CypB84–9G	KFHRVIKDG	0	700	270	0	150	150	450	2/7
ART4 ₁₃ original	AFLRHAAL	360		1120	195	500			
ART413–1G	GFLRHAAL	120		500	195	140			0/4
ART413–2G	AGLRHAAL	0		0	0	0			4/4
ART413–3G	AFGRHAAL	168		375	180	81			1/4
ART413–4G	AFLGHAAL	0		0	0	0			4/4
ART413–5G	AFLRGAAL	150		225	140	192			1/4
ART413–6G	AFLRHGAL	360		225	140	228			1/4
ART413–7G	AFLRHAGL	180		500	750	300			0/4
ART413–8G	AFLRHAAG	120		360	500	80			1/4
Saline		0	0	0	0	0	0	0	

The results of the skin test are shown by the area of flare (mm × mm)

Less than 0.25 of flare area ratio (FR) was considered as being significantly suppressed, and these cases appear in bold typeface

Table 2

peptide were important for the binding of CypB₈₄-specific IgE in one case (HD6 of Table 2) (Fig. 3, upper left column). The importance of the aa at positions 7 and 9 was further confirmed by an inhibition assay (Fig. 3, upper right column). In three cases (HD10, 15, 40), aa residues at positions 1, 7 and 9 of the CypB₈₄ peptide were important for antibody recognition by the binding assay, and those at positions 1, 6, 7 and 9 were important for recognition by the inhi-

bition assay (data not shown). Amino acid residues at positions 1, 3, 5, 6 and 7, or positions 5, 6, 7 and 8 of the ART4₁₃ peptide were important for recognition by anti-ART4₁₃-specific IgE by the binding or inhibition assay, respectively, in one case (HD11 of Table 1) (Fig. 3, lower columns). In two cases (HD32, 40), aa residues at positions 1, 3, 6 and 7 of the ART4₁₃ peptide were important for antibody recognition by the binding assay, and those at positions 1,

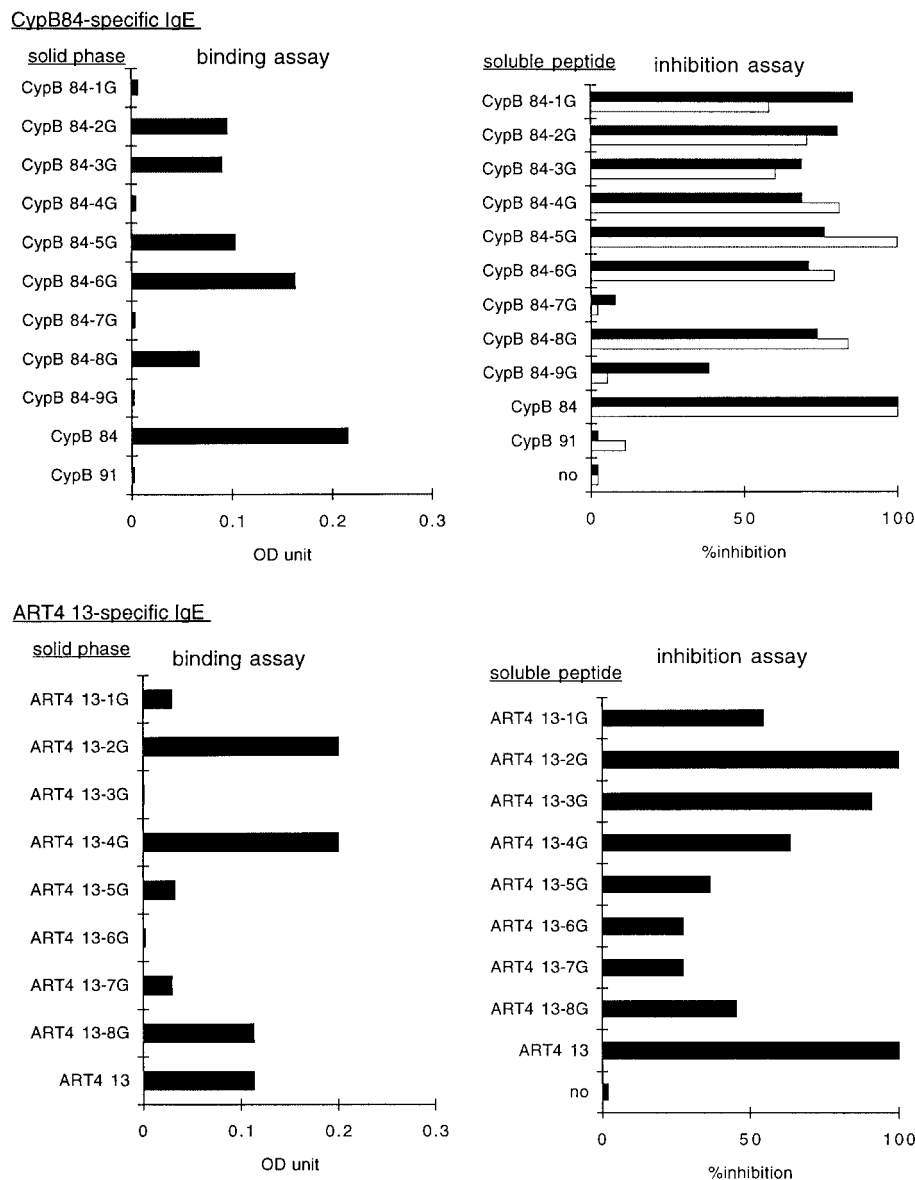


Fig. 3. Determination of amino acid (aa) residues of CypB₈₄ and ART4₁₃ peptides important for recognition by serum antibodies.

CypB₈₄- or ART4₁₃-specific IgE positive serum from HD6 or 11 was subjected to ELISA, and the results on CypB₈₄ or ART4₁₃ are shown in upper or lower columns, respectively. Glycine-substituted peptides of CypB₈₄ or ART4₁₃ were immobilized on the surface of a 96-well plate and were used for ELISA as an antigen-coated plate for the binding assay (left side figures). The soluble form of the glycine-substituted peptides was also used for the inhibition assay (right side figures). The concentration of the soluble peptides was 830 μ M (solid bar) and 166 μ M (open bar) for CypB₈₄-specific IgE, and 609 μ M (solid bar) for ART4₁₃-specific IgE.

6, 7 and 8 were important for recognition by the inhibition assay (data not shown). The lack of correlation between the binding and inhibition assays probably reflects differences in the antibody affinities detected in the two assays. Collectively, aa residues 7 and 9 of CypB₈₄ and those at positions 1, 6 and 7 of ART₄₁₃ were more important than the other aa residues for antibody recognition, respectively. However, there were no common aa residues important for both elicitation of skin reactions and serum antibody recognition in these healthy subjects. These results, which showed a large diversity of epitopic aa residues, could be a reflection of the polyclonality of peptide-specific IgE antibodies and might also indicate their heterogeneity among individuals (24–26).

Skin reactions to CypB₈₄ homologs from various species

CypB is a highly conserved molecule found in various species, including vertebrates, non-vertebrates, protozoans, plants and fungi (11, 15, 27–29). Both CypB₈₄ and CypB₉₁ homolog sequences are also found in these species and representative sequences of these CypB₈₄ homologs are shown in Table3. Skin reactions to the CypB₈₄ homologs were tested in five healthy volunteers to improve understanding of the involvement of non-self environmental antigen-derived peptides in the CypB₈₄-induced type-I allergy. Positive skin reactions to all of the CypB₈₄ homologs were observed in these volunteers, and the degree of skin reactions to these environmental homologs were for the most part equal to those to human CypB₈₄ (Table3). One exception was the *Streptomyces chrysomallus*-derived CypB₈₄ homolog, which induced skin reactions weaker than those to human CypB₈₄. In contrast to

CypB, to the best of our knowledge, homologs of human ART₄, SART2 and SART3 have not been identified at either the nucleotide or the protein level in any species other than mammals. Subsequently, unlike in the case of CypB, natural sensitization by microorganisms or foods may not largely affect IgE responses to the ART₄, SART2, and SART3 peptides shown in this study.

Kinetics of antibody production and allergic responses in patients receiving peptide vaccinations

The kinetics of the peptide-specific serum IgE and IgG levels and their correlation to type-I allergy in cancer patients receiving non-allergic peptide vaccinations were studied to improve understanding of the influence of vaccinations of nonallergic peptides on reactions to allergic neighborhood peptides. Representative results of three patients vaccinated with the CypB₉₁, ART₄₇₅ or SART₃₁₀₉ peptide are shown in Fig.4A-C, respectively. CypB₈₄, ART₄₁₃ and SART₃₁₅ elicited type-I allergy at prevaccination skin tests in these patients, and these peptides were not used for the vaccinations. Significant levels of IgE to CypB₈₄, ART₄₁₃ and SART₃₁₅ were detectable in the prevaccination sera, and these levels decreased in the postvaccination sera of patients that were vaccinated with nonallergic CypB₉₁, ART₄₇₅ and SART₃₁₀₉ peptide, respectively. In contrast, IgE to nonallergic CypB₉₁ or SART₃₁₀₉ were undetectable in prevaccination sera, and a low level of IgE to ART₄₇₅ was detected in the prevaccination serum. IgE to CypB₉₁ was undetectable in the sera of post (2 and 3 times) vaccination with CypB₉₁, whereas the levels of IgE to ART₄₇₅ increased in the sera of patients post (3, 9 and 15 times) vaccination

Allergic skin reaction to CypB₈₄ related peptides derived from various species

Origin of CypB	Human CypB ₈₄ homolog ^a	HD1	HD2	HD3	HD6	HD10
Human	KFHRVIKDF	600	2025	875	600	420
<i>Drosophila melanogaster</i>	KFHRIIKDF	400	1575	900	870	300
<i>Lumbricus rubellus</i>	KFHRVIPKF	600	750	1225	500	625
<i>Caenorhabditis elegans</i>	KFHRVIADF	100	925	625	450	0
<i>Arabidopsis thaliana</i>	SFHRIIKDF	600	2025	1350	270	396
<i>Leishmania major</i>	PFHRVIPDF	525	2000	1400	700	270
<i>Streptomyces chrysomallus</i>	SFHRVIKDF	0	400	225	255	100
<i>Aspergillus nidulleanus</i>	NFHRVIKDF	360	1400	600	1350	300
<i>Trichophyton mentagrophytes</i>	TFHRVIKDF	540	1400	750	450	300
<i>Malassezia furfur</i>	HFHRVIPDF	375	1225	750	900	156
Saline		0	0	0	0	0

^aAmino acid residues different from the sequence of human CypB₈₄ peptide appear in bold typeface
The results of the skin test with the CypB₈₄ homolog peptide are shown by the area of flare (mm × mm)

Table 3

with ART4₇₅, and IgE specific to SART3₁₀₉ became detectable in the sera of patients post (11 and 17 times) vaccination with SART3₁₀₉. It is of note that ART4₁₃ and SART3₃₁₅ failed to elicit a type-I allergy at prevaccination skin tests before the 9th and 17th vaccinations with the ART4₇₅ and SART3₁₀₉ peptides, respectively. Subsequently, the ART4₁₃ and SART3₃₁₅ peptides were safely given to cancer patients at the 9th and 17th vaccinations and also thereafter, respectively. These vaccinations with the two

ART4 and SART3 peptides did not induce any adverse effects, including immediate local or systemic hypersensitivities in the cancer patients. CypB₈₄ peptide still elicited type-I allergy at pre-vaccination skin tests before the 3rd vaccinations with CypB₉₁ peptide, although the degree of skin reaction was significantly decreased. No significant alteration in the distribution of subclass of total IgG or of peptide-specific IgG to any of these peptides was observed during the entire vaccination period (data not shown).

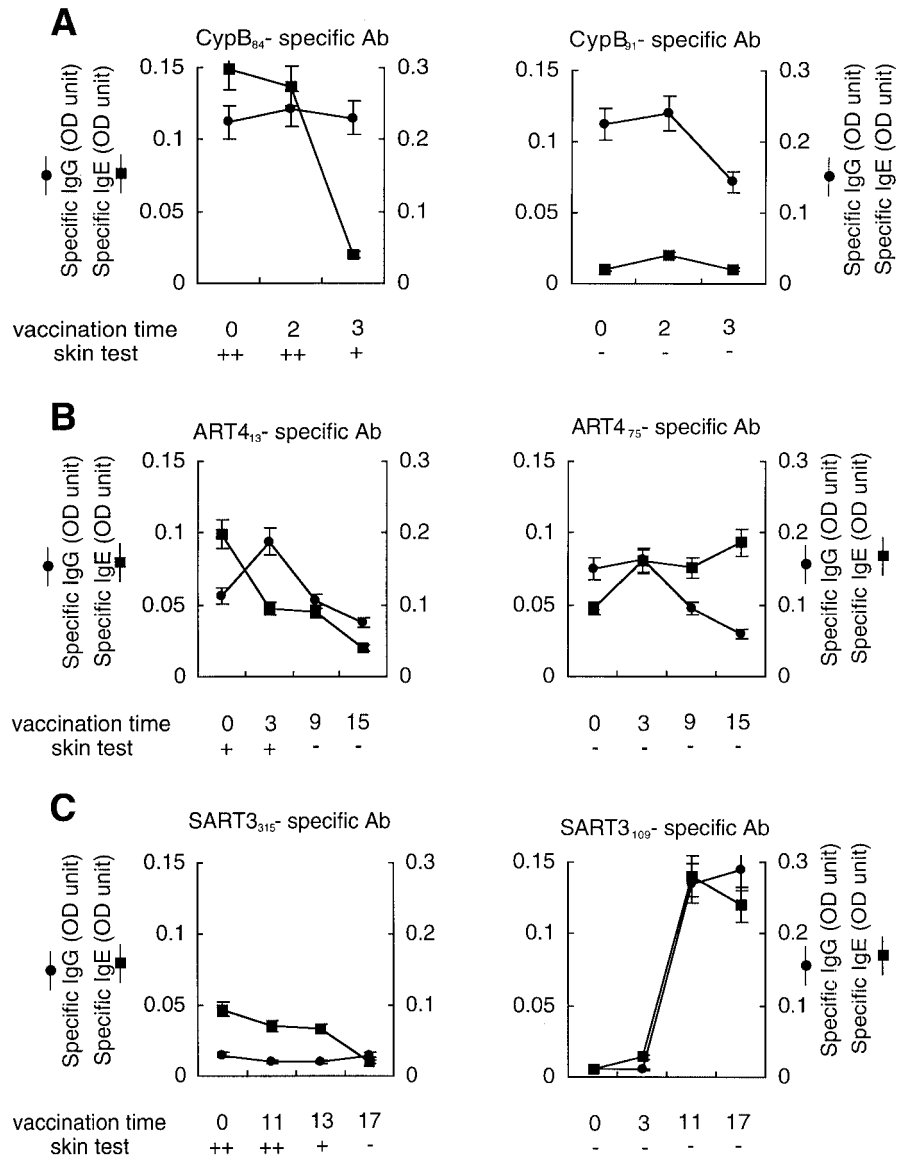


Fig. 4. The kinetics of antibodies and allergy in cancer patients receiving peptide vaccinations. (A) Sera from a lung cancer patient vaccinated with CypB₉₁ peptide were subjected to measurement of CypB₈₄- or CypB₉₁-specific IgE and IgG. (B) Sera from an ovarian cancer patient were obtained at different time points of vaccination with the ART4₇₅ peptide, and ART4₁₃- or ART4₇₅-specific IgE and IgG levels were then measured. (C) Sera from a colon cancer patient were obtained at different time points of vaccination with the SART3₁₀₉ peptide, and then SART3₁₀₉- or SART3₃₁₅-specific IgE and IgG levels were measured. The results of the skin test prior to each vaccination are also shown. Definition of the results of skin test was as follows: -- = longest diameter (D) of flare < 10 mm, + = 10 mm ≤ D < 30 mm, ++ = 30 mm ≤ D < 50 mm, +++ = D ≥ 50 mm.

Discussion

Autoantibodies to cell-surface antigens or nuclear components such as DNA and histone have often been detected in the sera of patients with autoimmune diseases and have also been detected in small amounts in the sera of healthy donors, most of whom had the IgM or IgG isotype (2, 30–33). Over last decade, large numbers of self-antigens recognized by both the CTLs and the IgG from the sera of cancer patients have been identified by cDNA expression cloning methods and serological analysis of recombinant cDNA expression (SEREX) methods, respectively (13–20, 34, 35). Furthermore, there is accumulating evidence that IgE-mediated responses to self-antigens could play a role in the pathogenesis of severe atopic diseases, based upon the cloning and sequencing of cDNAs encoding allergic molecules (4–12, 21). The results of the present study of healthy individuals and those of the patients may provide new insights to improve understanding of the immunological and pathological significance of auto-reactivity to self-antigens in the host immune surveillance against cancer and hypersensitivity diseases. However, the biological significance of both the specific IgE response and the type-I allergy to non-mutated self-peptides recognized by CTLs in healthy donors is unclear at the present time.

Our recent studies on the sera of patients with atopic dermatitis have revealed two interesting points: (i) serum IgE and IgG specific to allergic self-peptides in atopic dermatitis patients were significantly higher than those in healthy individuals and cancer patients, and (ii) in atopic dermatitis patients, the pattern of serum IgE specific to non-mutated self-peptides differed greatly from that of healthy individuals and cancer patients (Ohkouchi et al. unpublished results). Further studies will be needed to clarify the biological significance of specific IgE responses and type-I allergy to non-mutated self-peptides in healthy individuals, patients with atopic dermatitis and cancer patients.

It has been reported that IgE antibodies from patients sensitized to environmental allergens such as *Aspergillus fumigatus* cross-reacted with human mitochondria manganese super oxide dismutase (MnSOD), profilin, P2 ribosomal protein, cyclophilin B and SART1 (7–12, 21). However, allergic skin reactions and specific IgE to the self-peptides detected in healthy donors may not be primarily directed by environmental allergens, because: (i) all of the healthy individuals in this study were free from atopic diseases, (ii) serum IgE levels in response to the *Aspergillus* allergen, tics or household dust did not correlate with those to self-peptides (data not shown),

and (iii) ART4, SART2 and SART3 homologs are not found in fungi or other environmental microorganisms.

The cross-linking of IgE-bound high-affinity FcεR-I on mast cells by allergens and subsequent degranulation is a major pathway for allergic skin reactions (2, 37). In the present study, we tested such allergic reactions using 9-mer peptides. The mechanisms by which such a small peptide elicits an allergic reaction might be similar to those of small-molecular weight antibiotics or other drug-induced reactions (38). More specifically, the peptide molecules may form a complex with serum proteins or extra cellular matrix proteins, or may bind to the cell surface after intradermal injection. The complex, rather than being an aggregated form of the peptides, cross-links with FcεR-I on mast cells. The ineffectiveness of ultracentrifugation in removing the aggregated form in relation to the ability to elicit allergic skin reactions supports this hypothesis (data not shown). The binding activity of the peptides to HLA-A or DR molecules seems unrelated to the formation of the complex, as there was no obvious correlation between the allergic reactions to the peptides and the HLA-A or -DR types of the present subjects.

Suppression of both allergic peptide-specific IgE responses and immediate skin reactions by repeated vaccinations with a nonallergenic neighborhood peptide derived from the same protein may provide a new tool for the manipulation of allergic diseases. Conventional desensitization using whole allergen extracts is always associated with the risk of systemic anaphylaxis (38). As an alternative, allergen-specific desensitization by immunization with class-II restricted peptides recognized by CD4⁺ T cells has been attempted (39–43). However, such class-II restricted peptides have the potential to enhance not only Th1 but also Th2 responses in vaccinated hosts. In the course of developing peptide-based specific immunotherapy for cancer patients, we observed that repeated vaccinations with nonallergenic self-peptides recognized by the HLA class I-restricted CTLs suppressed both the specific IgE responses and the immediate skin reactions to allergenic peptides derived from the same protein molecule. One explanation for this would be that repeated vaccinations with class I-restricted and nonallergic peptide induced a clonal expansion of the nonallergenic peptide-specific CD8⁺ T cells, which in turn suppressed the pre-existing specific IgE responses to allergenic neighborhood peptides. Although the mechanisms are unknown at the present time, these results may provide new evidence for the development of allergen-specific desensitization by immunization with class-I restricted and nonallergic neighborhood peptides for allergic diseases.

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