



Peptide array-based analysis of the specific IgE and IgG4 in cow's milk allergens and its use in allergy evaluation

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ABSTRACT

Cow's milk (CM) is one of the major causes of food allergies in children. We constructed a peptide array consisting of a linear 16-mer peptide library with an offset of 3-mer, which corresponds to the primary sequences of six major CM allergens. The immune reactivity to cow's milk proteins diminishes with age and clinical tolerance commonly occurs. Although the central role of IgE in allergy is well established, the role of other specific antibody classes in obtaining immunotolerance is not well known. The hypothesis that patients become tolerant when they develop immunological changes particularly with the IgG4 isotype has been proposed. In this study, the binding pattern of the CM protein-specific IgE and IgG4 epitopes was measured using the peptide array with sera of 12 patients with persistent CM allergy (CMA), sera of 5 children who outgrew CMA, and sera of 7 CM-sensitized children without allergy symptoms. In CMA patients the IgG4/IgE fluorescence intensity ratios varied greatly from peptide to peptide, and the scatter plots of IgE versus IgG4 signals using significant IgE-binding peptides showed different distribution patterns. When setting the boundary line based on the IgG4/IgE ratio (IgG4/IgE = 2), patients with persistent CMA and CM-sensitized children can be distinguished by the plot pattern of peptides. Furthermore, the number of peptide plots in these regions was less in children who outgrew CMA. The approach employed in this study will allow for the distinction between CMA and CM-sensitization, and will enable the estimation of CMA outgrow by monitoring the time elapsed data.

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1. Introduction

Recent advances in the microfabrication process and surface modification technologies enable the construction of the peptide microarray format to be common tools for high throughput screening. Peptide arrays have become powerful tools for the evaluation of ligand–receptor interactions in a high throughput manner and have accelerated the process of epitope mapping, enzyme substrate screening, and the screening of bioactive inhibitory peptides [8,16,18–20]. Also, peptide arrays have become

versatile tools for high throughput screening assays in biomedical and pharmaceutical research [1,17].

For evaluation of food allergies using the peptide array, previous studies showed that the development of significant quantities of IgE antibodies against linear epitopes is associated with prolonged food hypersensitivity [12,33], and therefore it is important to identify the linear epitopes using a peptide array. The analysis of IgE levels specific to epitope peptides of ω -5 gliadin and high molecular weight–glutenin was suggested as a useful *in vitro* diagnostic tool for the assessment of patients with wheat-dependent exercise-induced anaphylaxis [15]. In peanut allergy, the diversity of epitope recognition was associated with the diversity of an allergic reaction [11]. Also, the differential recognition patterns of reactive and tolerant patients with cow's milk allergy were shown [4]. Thus, peptide array data could have prognostic value.

Cow's milk (CM) is one of the major causes of food allergies in children, and the immune mechanisms causing cow's milk allergy (CMA) can be classified into the IgE-mediated and non-IgE-mediated groups. The severity of CMA symptoms varies from one

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patient to another, and can include inflammation of the skin, gastrointestinal tract, and respiratory tract. The immune reactivity to cow's milk proteins (CMP) diminishes with age and clinical tolerance commonly occurs. In children younger than 4 years of age, the remission rate is lower in those with IgE-mediated CMA than in those with a non-IgE-mediated reaction [34], and recent studies have indicated that approximately 80% of children with an IgE-mediated reaction are still allergic at the age of 4, 60% at the age of 8, and 20% at the age of 16 [25]. The mainstay for treating milk allergy consists of prescribing the dietary elimination of cow's milk and having regular follow-up visits to re-evaluate the status of the allergy [31].

IgE-mediated reactions (type I allergic reactions) such as CMA are triggered by the binding of serum allergen-specific IgE antibodies to high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophiles, when the cross-linking of the receptors by multivalent allergens results in the release of biologically active substances such as histamine in the granules, i.e. degranulation [30]. Although the central role of IgE is well established, the role of other specific antibody classes in obtaining immunotolerance is not well known. The hypothesis that patients become tolerant when they develop immunological changes particularly with the IgG4 isotype has been reported; allergen-specific IgG, particularly the IgG4 isotype, is produced in high levels [28]. The production of IgE and IgG4 antibodies is regulated by similar mechanisms, e.g. Interleukin (IL)-4 from Th2 cells induces both IgE and IgG4 switching in B-cells, and these are mediated by an increase in allergen-induced IL-10, which is involved in the counter-regulation of these immunoglobulin responses [32]. Hence, it would be of interest to compare CMP-specific IgG4 responses between patients with IgE-mediated CMA and tolerant patients.

Although some laboratory tests for determining protein levels have been used in allergy prognosis, problems such as highly frequent false positive and false negative results are associated with these tests [22,23]. Moreover, CMP-specific IgE and IgG4 levels were also compared in CMA and tolerant patients [21,26,29]; however, their diagnostic value is controversial. The ELISA tests currently examined in clinical laboratories detect allergen-specific IgE antibodies, these tests do not give as further information on the number of epitopes and IgE-binding patterns that are responsible to induce allergic reactions, because the purified whole proteins were used as allergen to detect allergen-specific IgE antibody levels under current ELISA tests. Using the peptide array, we can get more precise data on allergens detected by specific IgE antibodies. The consideration of IgE and IgG4 epitope analyses using the peptide array might permit a more detailed assessment and precise diagnosis by obtaining more accurate information, such as an evaluation of epitopes related to the allergic reactions, to understand the clinical tolerance of the allergy or the severity of the allergy symptoms.

We recently constructed a peptide array containing 31 major epitopes of cow's milk allergens using a ceramic piezoelectric micropump and a conventional immunofluorescent assay, which was used to quantify epitopes of allergen-specific IgE [14]. In the present study, we developed a peptide array consisting of a library of linear 16-mer peptides with an offset of 3-mer, corresponding to the primary sequences of 6 major cow's milk allergens: α-lactalbumin (ALA), β-lactoglobulin (BLG), and caseins (α_{s1}-, α_{s2}-, β-, and κ-casein). The peptide array contained 605 peptides related to CMP were spotted in triplicate by ceramic piezoelectric micropump. Analysis of IgE and IgG4-binding sites with persistent CMA patients that is suspected to have serious symptoms in cow's milk, the milk-tolerant children that previously have CMA, and the sensitized children without CMA, will provide important information in characterization of IgE and IgG-binding sites related to CMA and CMA outgrow. The aim of this study is to detect the changes in

the amount of CMP-specific IgE and IgG4 using the peptide array and thus to categorized patients. The accumulation of monitoring data would further provide clinically valuable epitope peptides for diagnosis and further construction of tailor-made medicines.

2. Materials and methods

2.1. Reagents and materials

Succinimidyl active ester-modified glass slides (Geneslide) and micro-cover glass rectangles (size 24 mm × 60 mm, thickness 0.35–0.45 mm) were obtained from Toyo Kohan (Tokyo, Japan) and Matsunami Glass (Osaka, Japan), respectively. α-Lactalbumin (ALA), β-lactoglobulin (BLG), α-casein (AC), β-casein (BC), κ-casein (KC), and bovine serum albumin (BSA) were purchased from Sigma Chemical (St Louis, MO, USA). Human myeloma IgE and IgG4 was purchased from Chemicon (Temecula, CA, USA) and Calbiochem (San Diego, CA, USA), respectively. Goat anti-human IgE and sheep anti-human IgG4 polyclonal antibodies for spotting were purchased from Calbiochem and The Binding Site (Birmingham, West Midlands, UK), respectively. Goat anti-human IgE polyclonal and mouse anti-human IgG4 monoclonal antibodies for detection were purchased from American Qualex Antibodies (La Mirada, CA, USA) and Calbiochem, respectively. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and poly-DL-alanine were purchased from Pierce Chemical (Rockford, IL, USA), and Sigma Chemical, respectively. Alexa Fluor 647 and 555 carboxylic acid succinimidyl esters were purchased from Molecular Probes (Johnson City, TN, USA). Linear peptides (16-mer, >70% purity) were commercially synthesized by Invitrogen (Carlsbad, CA, USA).

2.2. Patient sera

CMA patient sera were obtained from Aichi Children's Health and Medical Center (Aichi, Japan) and Nagoya University Hospital (Aichi, Japan) (Table 1). The sera from 12 patients were selected retrospectively on the basis of IgE-mediated CMA symptoms by clinically controlled milk challenge (*n* = 7) or accidental ingestion while on a milk-free diet (*n* = 5). The patients' ages at the time of sera collection ranged from 4 to 20 years, with an average age of 9. Among these, 8 patients (patient Nos. 36, 49, 51, 52, 72, 82, 91, and 103) had a history of severe immediate reaction at a low dose (≤10 ml cow's milk or corresponding dairy product) after age 5, and physicians still strongly suspected remaining seriously allergic to cow's milk. Their levels of milk-specific IgE antibodies ranged from 1.43 to over 100 kU/l (mean 38.48 kU/l), as measured by the CAP System FEIA (Phadia, Uppsala, Sweden). The other 4 patients had high milk-specific IgE levels (≥70.3 kU/l). Patient Nos. 42 and 84 had confirmed CMA symptoms within 6 months before screening, and patient Nos. 88 and 102 had no recent history of ingestion because they each had a history of anaphylaxis and were considered too high-risk to undergo the oral food challenge. Therefore, these patients were classified as having persistent CMA. The allergic symptoms varied among individuals and involved inflammation of the skin, gastrointestinal tract, and respiratory tract. All these symptoms were observed within 2 h after ingestion of milk.

In addition, 12 sera from milk-tolerant children who can ingest more than 30 ml of cow's milk were used in a recent study, and divided into two groups. One group consisted of 5 children who had outgrown CMA, and serum samples were obtained from each after the lost of sensitivity. The mean level of milk-specific IgE antibodies was 2.66 kU/l in this group. Another group consisted of 7 CM-sensitized children with cow's milk-specific IgE ≥ 0.73 kU/l, who consumed milk daily without allergic reactions. Control sera

Table 1

Patient population used in open and blind tests of this study.

Patient	Sex/age	Symptoms ^a	Total IgE (UA/ml)	Milk CAP IgE (UA/ml)
Open test				
Group 1: Persistent CMA patients				
36	F/10Y8M	Oas, C, S	595	12.8
42	M/4Y10M	U, V, S, Dy, C, A	304	70.3
49	M/6Y0M	Cu	490	42
51	M/12Y8M	U, Ap, V, Dy	12,784	>100
52	M/6Y9M	R, Oas, C	2246	62.9
72	M/9Y8M	R, Oas, A	1753	68
82	M/20Y4M	S	15,850	10.4
84	F/6Y4M	E, V	14,283	>100
88	F/4Y4M	U, A	1741	>100
91	M/12Y7M	U, E, Oas, Ch, Ap, D, Dy	291	1.43
102	F/4Y8M	U, R, E, Ch, Ap, S, Dy, A	1658	>100
103	F/11Y6M	U, R, Oas, Nm, S, Dy, A	6280	10.3
Group 2: Children with CMA outgrow				
11	M/5Y11M		1288	3.65
37	F/2Y1M		67	3.78
64	M/10Y3M		1575	1.27
73	F/1Y1M		13	<0.34
87	F/1Y2M		141	4.23
Group 3: Sensitized children without CMA				
40	M/2Y9M		2392	3.94
79	M/5Y7M		1758	1.03
105	F/10M		153	1.06
106	F/7M		42.7	0.91
107	M/9M		229	0.73
108	M/1Y4M		344	1.1
109	M/11M		144	1.17
Blind test				
Group 1: Persistent CMA patients				
122	F/8Y11M	U, R, Oas, Ap, C, S, Dy, A	888	12.1
136	M/5Y11M	R, C, A	1237	65
152	M/12Y9M	U, E, S, Dy, A	595	12.6
Group 3: Sensitized children without CMA				
137	M/7Y10M		66.1	9.69
157	M/3Y8M		1334	1.88
159	M/14Y2M		27,815	3.959

^a U, urticaria; R, reddening; E, edema; Cu, contact urticaria; Oas, oral allergy syndrome; Ch, conjunctival hyperemia; Ap, abdominal pain; D, diarrhea; V, vomiting; Nm, nasal mucus; C, cough; S, stridor; Dy, dyspnoea; C, cyanosis; A, anaphylaxis.

were obtained from non-milk-allergic children with cow's milk-specific IgE < 0.35 kU/l.

In the blind test, 3 persistent CMA patients' sera were used, and the mean age of these patients was 9 years. Patient Nos. 122 and 152 had a documented history of severe immediate reaction at a low dose (≤ 10 ml cow's milk or dairy product) after age 5, and their mean level of milk-specific IgE antibodies was 12.35 kU/l. Patient No. 136 had high milk-specific IgE level (65 kU/l), and had a history of anaphylaxis. Three sera from CM-sensitized children who consumed milk daily without allergic reactions were also used, and the levels of cow's milk-specific IgE were ≥ 1.88 kU/l.

To monitor the time elapsed data from CMA to CMA outgrown, the sera from a patient who had confirmed CMA symptoms by accidental ingestion at the age of 9 months and CMA outgrown by clinically controlled milk challenge at the age of 1.5 while on a milk-free diet were used at 4 time points (3, 5, 9, and 18 months). The patients provided informed consent, and the protocols were approved by the local institutional review board.

2.3. Preparation of peptide arrays

A library of peptides used in this study consists of 16 amino acids overlapping by 13 (3-offset), and corresponds to the primary sequences of ALA (UniprotKB P00711), BLG (UniprotKB P02754),

and caseins (α_{s1} - (UniprotKB P02662), α_{s2} - (UniprotKB P02663), β - (UniprotKB P02666), and κ -casein (UniprotKB P02668)). In addition, amino acid differences in the genetic variants of each protein are also covered [10], although further analysis in this study was not used. All 608 commercially synthesized linear peptides (605 CMP-derived peptides and 3 negative control peptides (poly-DL-alanine, 4-mer poly-glycine, and angiotensin II)) were individually dissolved in a 20 mM phosphate buffer (pH 8.5) containing 0.1% (w/v) sodium dodecyl sulfate (SDS). After the addition of EDC, peptide solutions were spotted in at least triplicate on a succinimidyl-functionalized glass slide to a final spot density of 1947 spots per slide using the piezoelectric ceramic micropump (GENESHOT, NGK Insulators, Ltd., Aichi, Japan). The vacuum-packed peptide arrays were stored in desiccators at room temperature.

2.4. Spotting of cow's milk proteins

Cow's milk proteins were dissolved in a 50 mM phosphate buffer (pH 8.5) to a final concentration of 100 μ g/ml. After the addition of EDC, these proteins were spotted in triplicate on the peptide array. Human myeloma IgE (10 μ g/ml), goat anti-human IgE (2.5 μ g/ml), human myeloma IgG4 (100 μ g/ml), and sheep anti-human IgG4 (40 μ g/ml) diluted in the same buffer were used as the positive controls. After spotting, the peptide arrays were kept for 1 h at room temperature. Free N-hydroxysuccinimide (NHS) was blocked on the slides by incubation in the blocking buffer containing 50 mM ethanolamine for 90 min. Then the slides were washed 3 times in PBS (pH 7.4) containing 0.1% (v/v) Tween 20 (PBS-T).

2.5. IgE-binding analysis

Blood sera were diluted 1:10 (v/v) in PBS-T containing 1% (w/v) ovalbumin to a final volume of 200 μ l. The diluted sera were then transferred to the surface of the slides and covered by a micro cover glass. The slides were incubated in a humid chamber and allowed to react with peptide and protein spots for 1 h at 37 °C, followed by overnight incubation at 4 °C. Then, the peptide array was washed 3 times for 5 min with PBS-T. Specific human IgE and IgG4 were detected by incubation with Alexa 647-labeled anti-human IgE diluted 1:500 (v/v) combined with Alexa 555-labeled anti-human IgG4 diluted 1:500 (v/v) in PBS-T containing 1% ovalbumin for 3 h. The secondary antibodies were previously prepared from anti-human IgE and anti-human IgG4 by labeling with Alexa Fluor 647- and 555-NHS, respectively, in accordance with the manufacturer's instruction. The labeled antibodies were purified on an NAP-5 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The peptide arrays were finally washed 3 times in PBS-T and in water for 5 min, and were dried by centrifugation at 800 rpm for 3 min. All these steps were performed at room temperature, unless otherwise indicated.

2.6. Signal detection and data analysis

An Agilent microarray scanner (model G2505B, software G2565BA/DA; Agilent Technologies, Palo Alto, CA, USA) was used to analyze the fluorescence images of Alexa 647-labeled anti-human IgE and Alexa 555-labeled anti-human IgG4 at an emission wavelength of 665 and 565 nm, respectively. Fluorescence intensities were quantified with GenePix Pro (Axon, Union City, CA, USA). The mean background-subtracted fluorescent intensity of intra-assay triplicates for each peptide was used for further analysis. For calculation of percent positive values for each peptide, cutoff values for IgE and IgG4 were determined from data from the 3 control sera at the 95th percentile of fluorescence intensity against all peptides. The maximum binding capacity was

70,000 AU for IgE and IgG4 in fluorescent intensity, although the capacity is still not checked for other immunoglobulin class such as IgM or IgA.

3. Results

3.1. IgE and IgG4-binding analysis using the peptide array for CMA analysis

We developed a peptide array consisting of a library of linear 16-mer peptides with an offset of 3-mer, corresponding to the primary sequences of 6 major cow's milk allergens: α -lactalbumin, β -lactoglobulin, and caseins (α_{s1} -, α_{s2} -, β -, and κ -casein). Fig. 1 shows a fluorescent image of the peptide array for IgE-binding analysis. As the peptide array consisted of triplicate spots for each peptide, the triplicate patterns were kept at the similar level (dashed square). The mean coefficient variation (CV) of IgE and IgG4 signals with fluorescence intensities above the lower limit of detection ($S/N = 2$) were 9.5% and 11.1% of patient No. 102's sera, respectively. Considering the previous study in which the mean CV (28%) had been evaluated for the affinity measurements in the peptide microarray manufactured by robot-supported immobilization of pre-synthesized peptides, these values were sufficiently low [27]. In

the present study, we fabricated 300 peptide microarrays at one time using the piezoelectric ceramic micropump. This spotting method has been already established by DNA microarray fabrication and actually the mean CV was significantly low. Therefore, the developed peptide array enables the highly reproducible detection of the specific IgE- and IgG4-recognition sites.

For the evaluation of IgE- and IgG4-recognition peptides, the sera of 12 CMA patients were analyzed using the peptide array, and the spots with fluorescence intensities above the lower limit of detection ($S/N = 2$) by immunoassay without serum were excluded from the analysis. Fig. 2 shows the averaged fluorescence intensity for IgE and IgG4 for each of the synthetic peptides of BLG, ALA, aS1C, aS2C, BC, and KC in 12 CMA patients. A strong reaction was observed with aS1C-derived peptides, especially carboxy-terminal site of the sequence, and BC-derived peptides. Although the IgE levels were relatively high in each peptide, some spots were recognized by IgG4 stronger than by IgE. The averaged fluorescence intensity for IgE and IgG4 of AC protein was the highest of all in protein spots (data not shown). This result is consistent with the finding of casein's strong antigenicity, particularly aS1C [5,9,21], and the carboxy-terminal end of aS1C (amino acids 173–194) has been also proposed as a predictive maker of a persistent allergy [5]. In BLG-, aS2C-, KC-derived peptides, the amount of IgE bound to

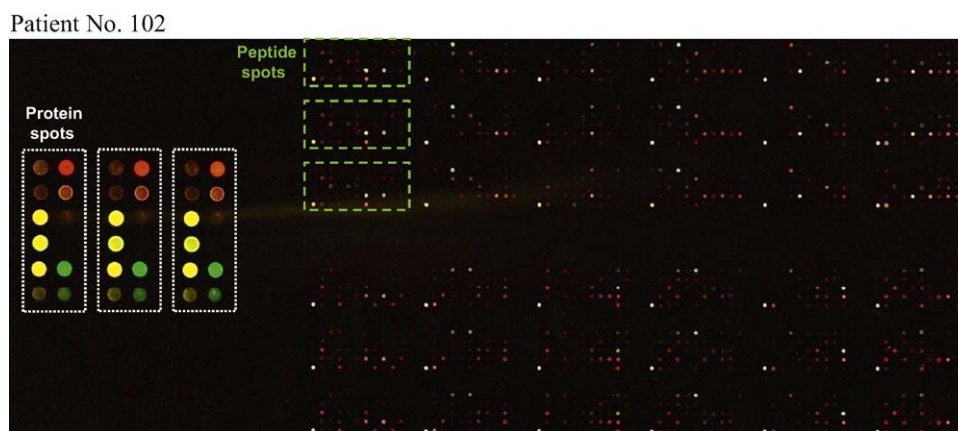


Fig. 1. Fluorescence images of the peptide array after immunofluorescent assays. As a representative example, the images of peptide arrays after immunofluorescent assay using patient No. 102's serum in CMA patient group are shown. Peptides and proteins were spotted in at least triplicate (dashed and dotted squares, respectively). Captured IgE and IgG4 antibodies against CMP-derived peptides were visualized with Alexa 647-labeled anti-human IgE (red color) and Alexa 555-labeled anti-human IgG4 secondary antibodies (green color), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

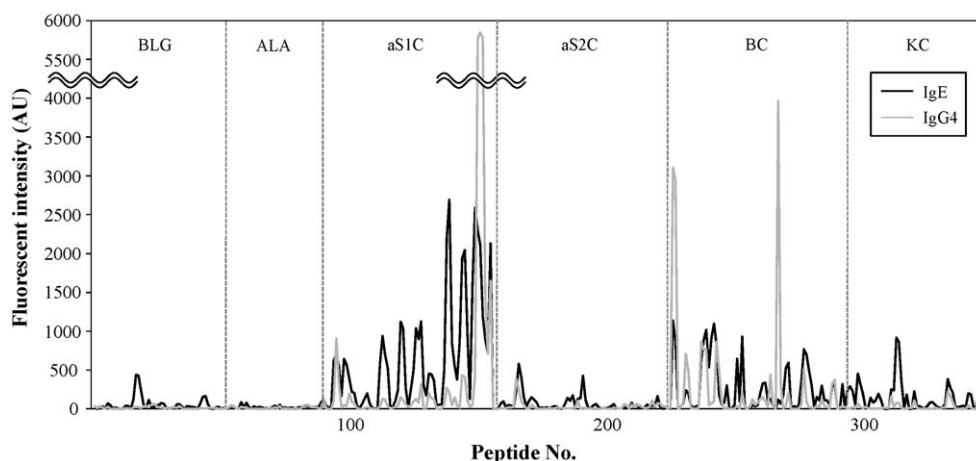


Fig. 2. Average fluorescence intensity of IgE and IgG4 for each of the synthetic peptides of CMP in CMA patient group. Sera from 12 CMA patients were used to evaluate IgE- and IgG4-binding pattern in 6 CMP (BLG, ALA, aS1C, aS2C, BC, and KC). Mean fluorescence intensity for binding of patient IgE (black line, $n = 12$) versus IgG4 (grey line, $n = 12$) is shown.

the same peptides was higher than that of IgG4. In protein spots, the binding to KC protein was stronger than that to BC protein. This is because KC has been shown to possess more tertiary structure [7], thus increasing the likelihood that it bears significant conformational epitopes when compared with BC (data not shown). ALA is also one of major antigens in CM, however, the reactivity against ALA-derived peptides and protein was hardly observed. Consequently, there was a strong correlation between data from the peptide and protein arrays, and the IgG4/IgE fluorescence intensity ratios varied greatly from peptide to peptide in CMA patients.

3.2. IgE and IgG4 scatter plots analysis

Fig. 3 shows the scatter plots of IgE versus IgG4 signal obtained from immunoassay of sera of patients with persistent CMA (A), children who have recently outgrown their CMA (B), and CM-sensitized children (C). In the CMA group, the single positive rate for the IgE region was 7.5% for all potential 348×12 CMP-derived peptides (the fluorescence intensities above the arbitrary cutoff were regarded as positive signals; see Section 2 for signal detection and data analysis). The double positive rate for IgG4/IgE region was highest in 8.0% of cases while the single positive rates for IgE region were lowest in 1.8% of cases in CMA outgrown and 1.2% in CM-sensitized children groups. The single positive rate for IgG4 was highest in CM-sensitized children (21.5% for all 348×7 peptides). Therefore, the scatter plots among groups showed obviously different distribution patterns, which allowed for the distinguishing between the groups.

3.3. Scattered distribution pattern of significant IgE-binding peptides with CMA sera

In the previous study, we discovered 73 significant IgE-binding peptides with CMA patient sera by comparative statistical analysis of 60 CMA patients and 22 non-milk-allergic children (including 20 CM-sensitized children with cow's milk-specific IgE ≥ 0.70 kU/l) using this peptide array. The identified peptides contained most of peptides derived from β -lactoglobulin (3 peptides), α -S1 casein (37 peptides), α -S2 casein (1 peptide), β -casein (22 peptides), and κ -casein (10 peptides), which is reported to be major antigen in milk proteins. It is highly possible that immunoglobulin against these peptides is associated with the clinical outcome of the allergy (unpublished data).

To characterize the distribution patterns in more detail, we consequently plotted IgE signal versus IgG4 signal in each group for all 73 significant IgE-binding peptides (Fig. 4). The double negative rates for the IgG4/IgE region were decreased in both CMA and CM-sensitized groups as compared to the data displayed in Fig. 3. As the tendency of a total signal, the single positive rate for IgE region and double positive rate for the IgG4/IgE region increased in the persistent CMA group, and the single positive rate for the IgG4 region and double positive rate for the IgG4/IgE region increased in the CM-sensitized group. We subsequently set the boundary line based on IgG4/IgE fluorescence intensity ratio (IgG4/IgE = 2) by a trial-and-error method to distinguish between these distribution patterns because the IgE and IgG4 signal highly skewed distribution patterns in IgG4/IgE double positive regions were found in the CMA and CM-sensitized groups, respectively. The distribution

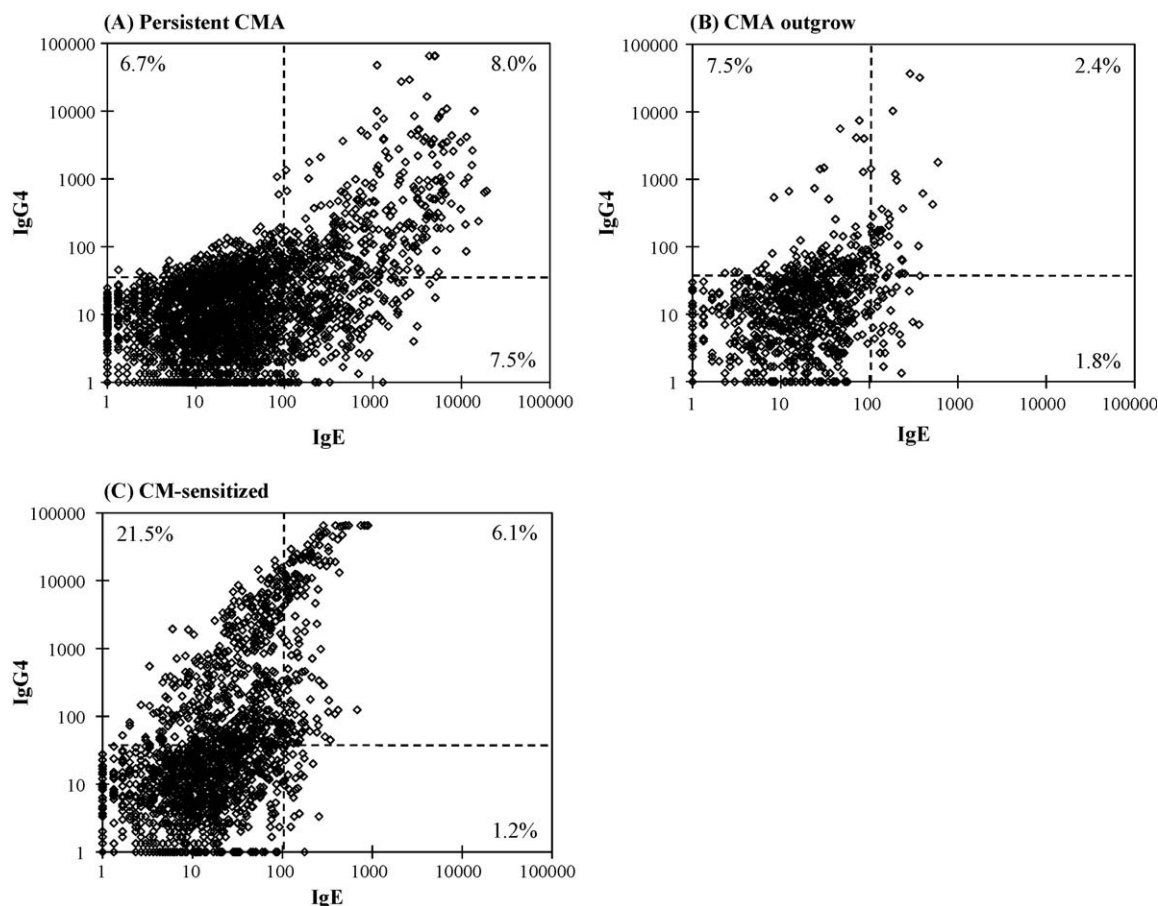


Fig. 3. Scatter plots of IgE versus IgG4 signal in persistent CMA (A), CMA outgrown (B), and CM-sensitized (C) groups. IgE versus IgG4 fluorescence intensity (log scale) for each peptide is shown in persistent CMA ($n = 12$), CMA outgrown ($n = 5$), and CM-sensitized group ($n = 7$). Dashed lines indicate cutoffs (see the Section 2). Numbers indicate a percentage above cutoff.

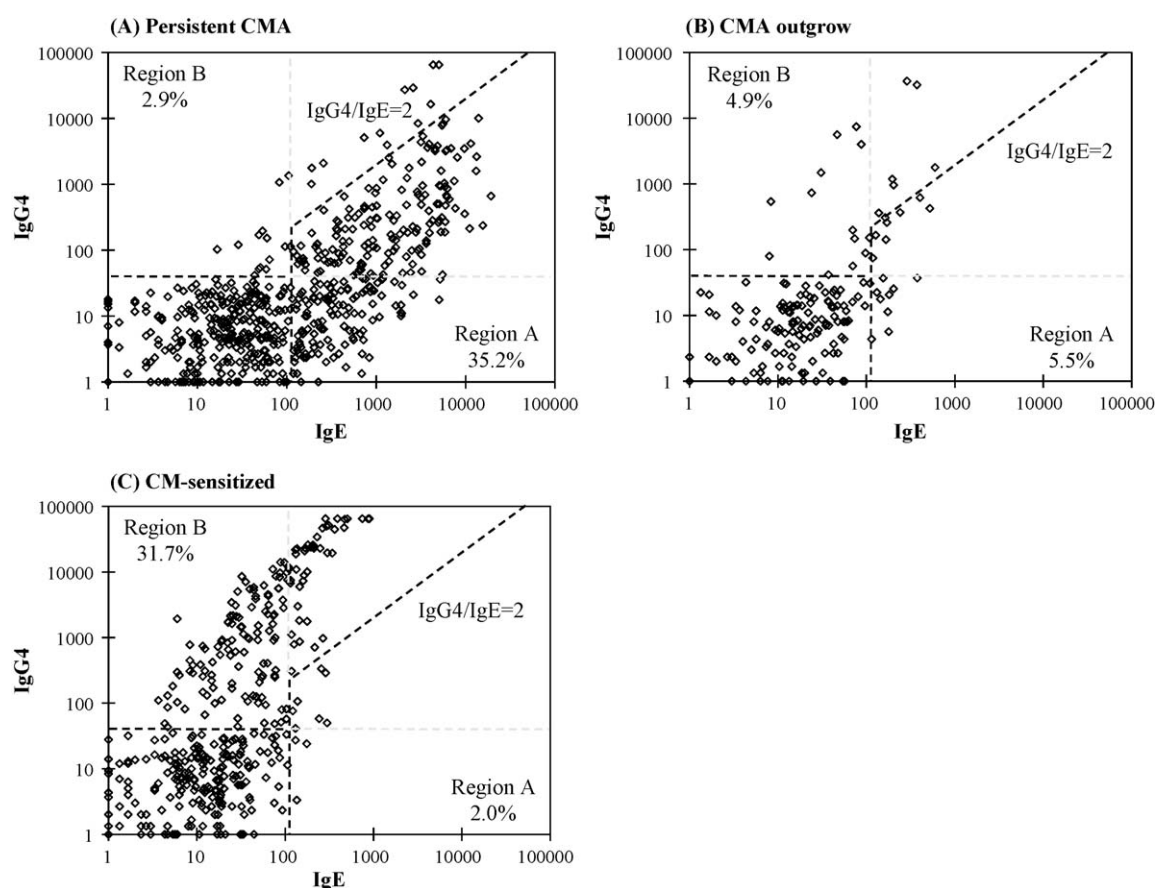


Fig. 4. Scatter plots of IgE versus IgG4 signal among groups using IgE-binding peptides. Scatter plots of IgE versus IgG4 signal by significant IgE-binding peptides for CMA patients are shown in persistent CMA ((A), $n = 12$), CMA outgrown ((B), $n = 5$), and CM-sensitized ((C), $n = 7$) groups. When setting the boundary line based on IgG4/IgE fluorescence intensity ratio ($\text{IgG4/IgE} = 2$, diagonal dashed line), regions A and B represented IgE single positive zone and $\text{IgG4/IgE} < 2$ in double positive zone, and $\text{IgG4/IgE} \geq 2$ in double positive zone and IgG4 single positive zone, respectively. The horizontal and vertical dashed lines indicate cutoffs (see Section 2). Numbers indicate a percentage in regions A and B.

rates in region A (IgE single positive zone and $\text{IgG4/IgE} < 2$ in double positive zone) were 35.2% and 2.0% in the CMA and CM-sensitized groups, respectively, while the rates in region B (IgG4 single positive zone and $\text{IgG4/IgE} \geq 2$ in double positive zone) were 2.9% and 31.7% in the CMA and CM-sensitized groups, respectively. In the CMA outgrown group, both distribution rates in regions A and B were low in less than 5.5% of cases. Therefore, the comparison of distribution rates in regions A and B showed distinct patterns for CMA and CM-sensitized groups.

3.4. The number of peptides in the regions A and B in individual patients

Table 2 shows the number of peptides in each region in persistent CMA patients, outgrown children, and sensitized children. Although there was variability in individual patients, persistent CMA patients had more peptides in region A than in region B, while CM-sensitized children had more peptides in region B than in region A. Therefore, the analysis of the distribution of IgE and IgG4 may be used to distinguish CMA from CM-sensitization.

3.5. Blind test

We validated the described approach using new peptide array data from 3 persistent CMA patients and 3 CM-sensitized children. Table 3 shows the number of peptides in each region with newly visited individuals. The number of peptides in region A was higher than that in region B in all persistent CMA patients, while the

Table 2

The number of peptides in each region in individual patients.

Group	Patient No.	Region A (the number of peptides) ^a	Region B (the number of peptides) ^a
Persistent CMA	36	21	2
	42	16	3
	49	17	5
	51	8	1
	52	35	0
	72	34	2
	82	7	3
	84	47	1
	88	58	4
	91	2	0
CMA outgrow	102	58	4
	103	6	0
	11	0	1
	37	11	7
	64	1	7
CM-sensitized	73	0	0
	87	8	3
	40	0	30
	79	1	21
	105	4	5
	106	3	26
	107	1	63
	108	1	9
	109	0	8

^a In each persistent CMA patient ($n = 7$), outgrown child ($n = 5$), and sensitized child ($n = 7$), the number of peptides in regions A and B were represented.

Table 3

The number of peptides in each region in newly visited individuals (blind test).

Group	Patient No.	Region A (the number of peptides) ^a	Region B (the number of peptides) ^a
Persistent CMA	122	18	9
	136	32	22
	152	23	15
CM-sensitized	137	1	34
	157	1	55
	159	8	25

^a In each newly visited persistent CMA patient ($n = 3$), and sensitized child ($n = 3$), the number of peptides in regions A and B were represented.

Table 4

The change of number of peptides in each region with age from CMA to CMA outgrown.

Age	Status	Milk CAP IgE (UA/ml)	Region A (the number of peptides)	Region B (the number of peptides)
3M	CMA	10.8	25	5
5M	CMA	5.28	18	1
9M	CMA	3.49	13	0
1Y6M	CMA outgrown	0.71	2	9

number of peptides in region B was higher than that in region A in all CM-sensitized children. Thus, using peptide array data we can distinguish between persistent CMA and CM-sensitization, and such application of the peptide array can be also used to verify individual epitopes.

3.6. The change of IgG4/IgE ratio with age from CMA to CMA outgrown

Finally, we examined CMA outgrow by monitoring the time elapsed data of IgG4/IgE ratio for all 73 significant IgE-binding peptides. As shown in Table 4, the number of peptides in both regions (region A and B) were gradually decreased with age until the age of 9 months, and at the age of 1.5 when the outgrow of CMA was confirmed, the distribution pattern was dramatically changed (the numbers of peptides in regions A and B were changed from 13 to 2 and from 0 to 9, respectively). These results suggest that the peptide distribution pattern is consistent in the outgrown CMA (Fig. 4 and Table 2). This patient still had positive CAP IgE result after he had outgrown CMA, that means false-positive result. Our approach using IgG4/IgE ratio may estimate outgrow of CMA by monitoring the time elapsed data.

4. Discussion

The peptide array is a promising technology for the detection and/or characterization of antibodies against linear epitopes. Recently it attracted interest for its use in discovery-oriented basic biological research and for developing new clinical diagnostic [8,16,18–20]. In the previous study, we constructed a peptide array that contained 31 linear peptide fragments reported as IgE-binding epitopes for cow's milk allergy, and it has been documented as a reliable tool to describe sequential IgE epitopes on milk allergen [14].

In the present study, we developed a peptide array consisting of all the peptides with overlapping milk protein sequences (Fig. 1), which was also used as a diagnostic tool for epitope mapping to identify IgE- and IgG4-binding peptides (Fig. 2). We used only 20 μ l of serum sample for IgE or IgG4-binding analysis of 6 kinds of protein as well as 605 peptides using the developed peptide microarray. The small amount of serum used would be also the advantage of using peptide array over conventional immuno-fluorescent assay, since it is difficult to obtain sufficient amount of

serum from patients younger than 1 year. In previous studies, the development of significant quantities of IgE antibodies against linear epitopes was associated with prolonged food hypersensitivity [12,33], and therefore it is important to identify the linear epitopes using a peptide array. In these assays, it is possible that there are other classes of immunoglobulin molecules competing with IgE or IgG4-binding to epitopes on peptide array. IgG4 has been attracted attention by its ability to compete allergen–IgE interaction and it has been termed “blocking antibody”. Even if there are other immunoglobulin molecules bind to certain IgE- or IgG4-epitopes and their interaction is disturbed, this will be reflected to the induction of the allergy symptoms. Therefore, detection of the competing interaction including the influence of other subclasses of immunoglobulin is rather to be attractive and useful to accurate diagnose for allergy. An IgE-binding assay with peptides synthesized by a SPOT membrane synthesis was also reported for the IgE-binding epitopes against CMP [2,3,5,6,13] and the diversity of epitope recognition was associated with the diversity of an allergic reaction in other food allergies [11]. Thus, peptide array data could have prognostic value.

CAP-RAST, capsulated hydrophilic carrier polymer-radioallergo-sorbent test is an ELISA assay for allergen-specific IgE detection with high sensitivity. CAP-RAST has been widely used in diagnostics. However, a correlation between the IgE level of CAP and the severity of the allergic reaction is not always observed [22,23]. Some studies that tried to evaluate CMA versus tolerant patients by not only IgE but also IgG4 levels and their prognostic application report controversial finding [21,26,29]. As a matter of course, the finding of clinically valuable epitope peptides using a peptide array is important to minimize the occurrence of false positive and false negative results during the blood tests. Although the detailed interpretation of data obtained with such epitope peptides is important in tailor-made medicine, the integrated interpretation of such data is essential for a primary screening for expedient diagnosis. Our approach, consisting of the setting of a boundary line (IgG4/IgE = 2) using the significant IgE-binding peptides, provides a potent capability for the discrimination of CMA and CM-sensitization (Fig. 4, and Tables 2 and 3). The present study included persistent CMA patients who are older and have serious CMA. However, this approach is also applicable to younger patients with transient CMA.

During the analysis of each individual in the CMA outgrown group, data from patient No. 37, who can consume up to 38 ml of cow's milk but has allergy symptoms over a certain amount of intake, showed more peptides in region A; patient No. 87, who does not show allergy symptoms with 68 ml of cow's milk ingestion, also has more peptides in region A. Alternatively, data from patient No. 64, who can consume up to 200 ml of cow's milk and already had consumed cow's milk daily, showed more peptides in region B. In addition, data from patient No. 105 in the CM-sensitized group, who did not consume cow's milk daily, showed slightly more peptides in region B than in region A. Thus, the daily intake of cow's milk might increase the IgG4 level as suggested the elevation of IgG level by intake in the previous study [24]. Therefore, our approach might be useful for the evaluation of sensitization patterns required for immunotherapy, although the present study used static analysis among groups. In addition, the accumulation of monitoring data of the change with age from CMA to CMA outgrown will provide further information about the relationship between IgE and IgG4 in CMA versus tolerant status although only a patient was estimated in Table 4.

In conclusion, we constructed a peptide array consisting of a linear 16-mer peptide library with an offset of 3-mer, which corresponds to the primary sequences of 6 major CM allergens. In CMA patients the IgG4/IgE fluorescence intensity ratios varied greatly from peptide to peptide, and the scatter plots of IgE versus IgG4 signals using significant IgE-binding peptides showed

different distribution patterns. As indicated in Fig. 4, the setting of the boundary line based on the IgG4/IgE ratio enabled to distinguish CMA from CM-sensitization. This study represents a preliminary step toward finding better diagnostic tools. The approach chosen here might be used for the categorization of patients. It provides more specific data compared with the currently used blood test for determining protein level, and therefore could reduce the occurrence of false positive results.

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