

Development of peptide arrays for detection of IgE-binding epitopes in cow's milk allergens

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Peptide arrays have become versatile tools for high throughput screening assays in biomedical and pharmaceutical research. In this study, we constructed a peptide array that contained linear peptide fragments reported as IgE-binding epitopes for cow's milk allergy (CMA). Various peptides with different solubility in aqueous solutions were dissolved in the buffer solutions containing sodium dodecyl sulfate, and we achieved a consistent spotting of peptide solutions using a piezoelectric ceramic micropump. The IgE-binding patterns were successfully detected by observing the binding of Alexa 647-labeled anti-human IgE using sera from CMA patients. Our technique in this study will provide a potent capability for the development of a peptide array for mapping IgE-epitopes in milk proteins, and it will help researchers better understand the IgE-epitopes associated with the clinical outcome of CMA.

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[Key words: Peptide array; Piezoelectric ceramic micropump; Cow's milk allergy; IgE-binding epitopes; spotting buffer]

Recently, there has been a growing interest in biochips for use in exhaustive biomolecular interaction analyses or as diagnostic tools. The immobilization of biomolecules onto a chip surface has enabled us to analyze enormous quantity of biomolecular interaction data. Although DNA microarray technology has become a standard technique for DNA expression profile analysis, comparable analytical tools for proteins are not available to date. This is not only because it is difficult to obtain proteins exhaustively but also it is difficult to immobilize bioactive proteins in a stable manner. Peptide arrays have become powerful tools for the evaluation of ligand-receptor interactions in a high throughput manner (1, 2) and have accelerated the process of epitope mapping, enzyme substrate screening, and the screening of bioactive inhibitory peptides (3–5).

Food allergies, or food intolerances, are a common medical problem. The prevalence of food allergies is increasing in industrialized countries, especially in children. The percentage of infants with food allergies is highest at age one and expected to rise by up to 8%, and then progressively fall until late childhood, after which the percentage is 1–2% (6). The current blood tests used widely in food allergies are based on the detection of

allergen-specific IgE. However, there are reports indicating that the amount of allergen-specific IgE antibody does not always correlate to subjective symptoms (7).

Cow's milk allergy (CMA) is one of the major causes of food allergies in children. The severity of CMA symptoms varies from one patient to another, and CMA symptoms involve inflammation of the skin, gastrointestinal tract, and respiratory tract. CMA is mainly an IgE-mediated hypersensitivity reaction, and recent studies have indicated that about 80% of children with an IgE-mediated reaction are still allergic at the age of four, 60% at the age of eight, and 20% at the age of 16 (8). Cow's milk proteins (CMP) are divided in two main classes: coagulum (curd; 80% of CMP) and lactoserum (whey; 20% of CMP). The major allergens of coagulum and lactoserum are four caseins (α S1-, α S2-, β -, and κ -casein), and β -lactoglobulin (BLG) and α -lactalbumin (ALA), respectively (9, 10). An IgE-binding assay, with peptides synthesized by a SPOT membrane synthesis, was reported for the IgE-binding epitopes (11–15). In persistent CMA, the significant enhancement of IgE production against linear epitopes in milk allergens was also suggested (16). However, there is not a clear association between the IgE-epitopes and the induction of the allergy symptoms. Therefore, it is important to obtain more accurate information, such as an evaluation of linear epitopes related to the allergic reactions using a peptide array in each patient, to understand the clinical outcome of the allergy or the severity of the allergy symptoms.

In the present study, a synthetic 16-mer peptide array, previously reported as IgE-binding epitopes in CMP, was constructed using a

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TABLE 1. The sequence of synthesized linear peptides spotted on the array

No.	Origin	Sequence (N→C)	Reference
1	α_{s1} -casein	(AA19-34) <u>NLLRFFVAPFPEVFGK</u>	(13)
2	α_{s1} -casein	(AA67-82) <u>SSEIVPNSVEQKHIQ</u>	(11)
3	α_{s1} -casein	(AA109-124) <u>LEIVPNSAEERLHSMK</u>	(13)
4	α_{s1} -casein	(AA121-136) <u>HSMKEGIHAQQKEPMI</u>	(11)
5	α_{s1} -casein	(AA172-187) <u>QYTDAPSFSDIPNPIG</u>	(13)
6	α_{s1} -casein	(AA173-188) <u>YTDAPSFSDIPNPIGS</u>	(13)
7	α_{s2} -casein	(AA28-43) <u>INPSKENLCSTFCKEV</u>	(11)
8	α_{s2} -casein	(AA82-97) <u>LNEINQFYQKFPQYLO</u>	(12)
9	α_{s2} -casein	(AA142-157) <u>ESTEVFTKTKLTEEE</u>	(12)
10	α_{s2} -casein	(AA157-172) <u>EKNRLNLFKIKSQRYQ</u>	(12)
11	α_{s2} -casein	(AA169-184) <u>QRYQKFAFPQYLVTVY</u>	(11)
12	β -casein	(AA1-16) <u>RELEELNVPGEIVESL</u>	(14)
13	β -casein	(AA55-70) <u>TQSLVYPFGPIPNLSL</u>	(14)
14	β -casein	(AA76-91) <u>PLTQTPVVPVFPFLQPE</u>	(14)
15	β -casein	(AA106-121) <u>HKEMPFKYPVEPFTE</u>	(14)
16	β -casein	(AA130-145) <u>VENLHLPLLLQSWMH</u>	(14)
17	β -casein	(AA148-163) <u>HQPLPPTVMFPQSVL</u>	(14)
18	κ -casein	(AA10-25) <u>RCEKDERFFSDKIAY</u>	(14)
19	κ -casein	(AA25-40) <u>YIPQYVLSRYPSYGL</u>	(14)
20	κ -casein	(AA52-67) <u>NNQFLPYPPYAKPAAV</u>	(14)
21	κ -casein	(AA67-82) <u>VRSPAQLQWQVLSNT</u>	(14)
22	κ -casein	(AA100-115) <u>HPHLSFMAIPPKNQD</u>	(14)
23	κ -casein	(AA136-151) <u>TEAVESTVATLEDSP</u>	(14)
24	κ -casein	(AA154-169) <u>ESPPEINTVQVTSTAV</u>	(11)
25	α -lactalbumin	(AA1-16) <u>EQLTKCEVFRELKDLK</u>	(15)
26	α -lactalbumin	(AA10-25) <u>RELKDLKGYGGVSLPE</u>	(15)
27	α -lactalbumin	(AA88-103) <u>DIMCVKKILDKVGIN</u>	(15)
28	β -lactoglobulin	(AA67-82) <u>AQKKIIAEKTKIPAVF</u>	(15)
29	β -lactoglobulin	(AA76-91) <u>TKIPAVFKIDALNENK</u>	(15)
30	β -lactoglobulin	(AA127-142) <u>EVDDEALEKFDKALKA</u>	(15)
31	β -lactoglobulin	(AA136-151) <u>FDKALKALPMHIRLSF</u>	(15)
32*	α_{s2} -casein	(AA143-158) <u>STEVFTKTKLTEEEK</u>	(12)

Peptide sequences reported previously as IgE-binding epitopes were underlined. Peptide nos. 1–31 were used for the development of the peptide array.

*Peptide No. 32 which was not a candidate of epitope peptide was used only for spotting test and not spotted on the peptide array.

piezoelectric ceramic micropump to establish diagnostic tools for the measurement of allergen-specific IgE. The piezoelectric ceramic micropump enables controlled spotting of peptides on arrays, and provides reliable results for epitope mapping. Peptide arrays provide an accurate analysis of the association between IgE-epitope recognition patterns and the clinical outcome of CMA.

MATERIALS AND METHODS

Reagents and materials Succinimidyl-functionalized glass slides (Geneslide) and micro cover glass rectangles (size 24×60-mm², thickness 0.35–0.45 mm) were obtained from Toyo Kohan (Tokyo, Japan) and Matsunami Glass (Osaka, Japan), respectively. ALA, 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 was purchased from Chemicon (Temecula, CA, USA). Goat anti-human IgE polyclonal antibodies for spotting and detection were purchased from Calbiochem (San Diego, CA, USA) and American Qualex Antibodies (La Mirada, CA, USA), respectively. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), poly-DL-alanine, and Alexa Fluor 647 carboxylic acid succinimidyl esters were purchased from Pierce Chemical (Rockford, IL, USA), Sigma Chemical, and Molecular Probes (Johnson City, TN, USA), respectively. Unlabeled and fluorescein isothiocyanate (FITC) labeled peptides (16-mer, >70% purity) were commercially synthesized by Invitrogen (Carlsbad, CA, USA) and Sigma Genosys (The Woodlands, TX, USA), respectively.

Patient sera Four patients with CMA and three control sera were obtained from Aichi Children's Health and Medical Center (Aichi, Japan) and Nagoya University Hospital (Aichi, Japan). These patient sera (from patient nos. 2, 28, 29, and 31) were selected retrospectively on the basis of IgE-mediated CMA symptoms within a year before the blood was drawn. The patients' ages ranged from 2 to 5 years, with an average age of 3. Their levels of milk-specific IgE antibodies ranged from 40.5 to above 100 kU/L, as measured by the CAP system fluoro-enzyme immunoassay (CAP-radioallergosorbent test (RAST) FEIA) (Phadia, Uppsala, Sweden) (17). Their allergic symptoms varied among individuals and involved inflammation of the skin, gastrointestinal tract, and respiratory tract. Two pooled sera from milk allergic patients (CAP class 2–3 and 2–6) were also tested in this study. Control sera were obtained from non-milk-allergic children with cow's milk-specific IgE <0.35 kU/L, most of which had other

allergy disease. All of the subjects provided informed consent, and all of the protocols were approved by the local institutional review board.

Preparation of peptide arrays The peptides used in this study (Table 1) were reported as the IgE-epitopes, some of which (peptide nos. 1, 5, 6, 8, 9, 14, 17–20, 22, 26, 30, and 31) were included partial sequences of reported IgE-binding sites (11–15). In the spotting test, two FITC-labeled peptides (peptide nos. 10 and 32) were dissolved in 20 mM phosphate buffer (pH 8.5) containing 0.1% (w/v) sodium dodecyl sulfate (SDS). After EDC addition, two peptide solutions were spotted 255 times on a succinimidyl-functionalized glass slide to a final spot density of 510 spots per slide using the piezoelectric ceramic micropump (GENESHOT, NGK insulators, Ltd., Aichi, Japan). In the development of the peptide arrays, each 32 kinds of peptide solutions (peptide nos. 1–31, and a poly-DL-alanine peptide; negative control peptide) was spotted in triplicate on the slide to a final spot density of 99 spots/slide. The vacuum-packed peptide arrays were stored in desiccators at room temperature.

Spotting of cow's milk proteins Cow's milk proteins were dissolved in 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 (hIgE) and goat anti-human IgE (anti-hIgE) were used as the positive controls, and were diluted in the same buffer to a final concentration of 10 μ g/ml. After spotting, the peptide arrays were kept for 1 h at room temperature. Free N-hydroxysuccinimide (NHS) was blocked on the slides by incubating the slides in the blocking buffer containing 50 mM ethanolamine for 90 min. The slides were then washed 3 times in PBS (pH 7.4) containing 0.1% (v/v) Tween 20 (PBS-T).

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 at 37 °C. Then, the peptide array was washed 3 times for 5 min with PBS-T. Specific human IgE was detected by incubation with Alexa 647-labeled anti-human IgE that was diluted 1:500 (v/v) in PBS-T containing 1% ovalbumin for 3 h. The secondary antibodies were previously prepared from anti-human IgE by labeling with Alexa Fluor 647-NHS, according to the manufacturer's instruction. The labeled antibodies were purified on a NAP-5 column (GE Healthcare, Little Chalfont, Bucks, UK). The peptide arrays were finally washed 3 times in PBS-T and in water for 5 min, and were allowed to dry by

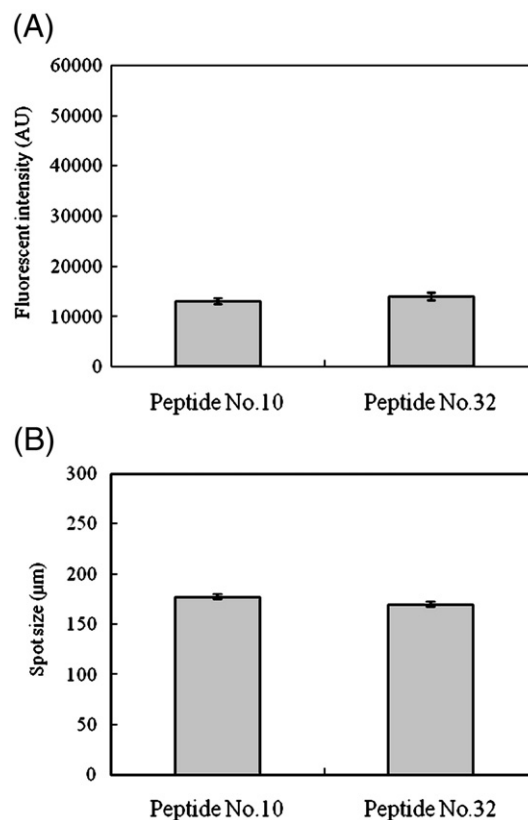
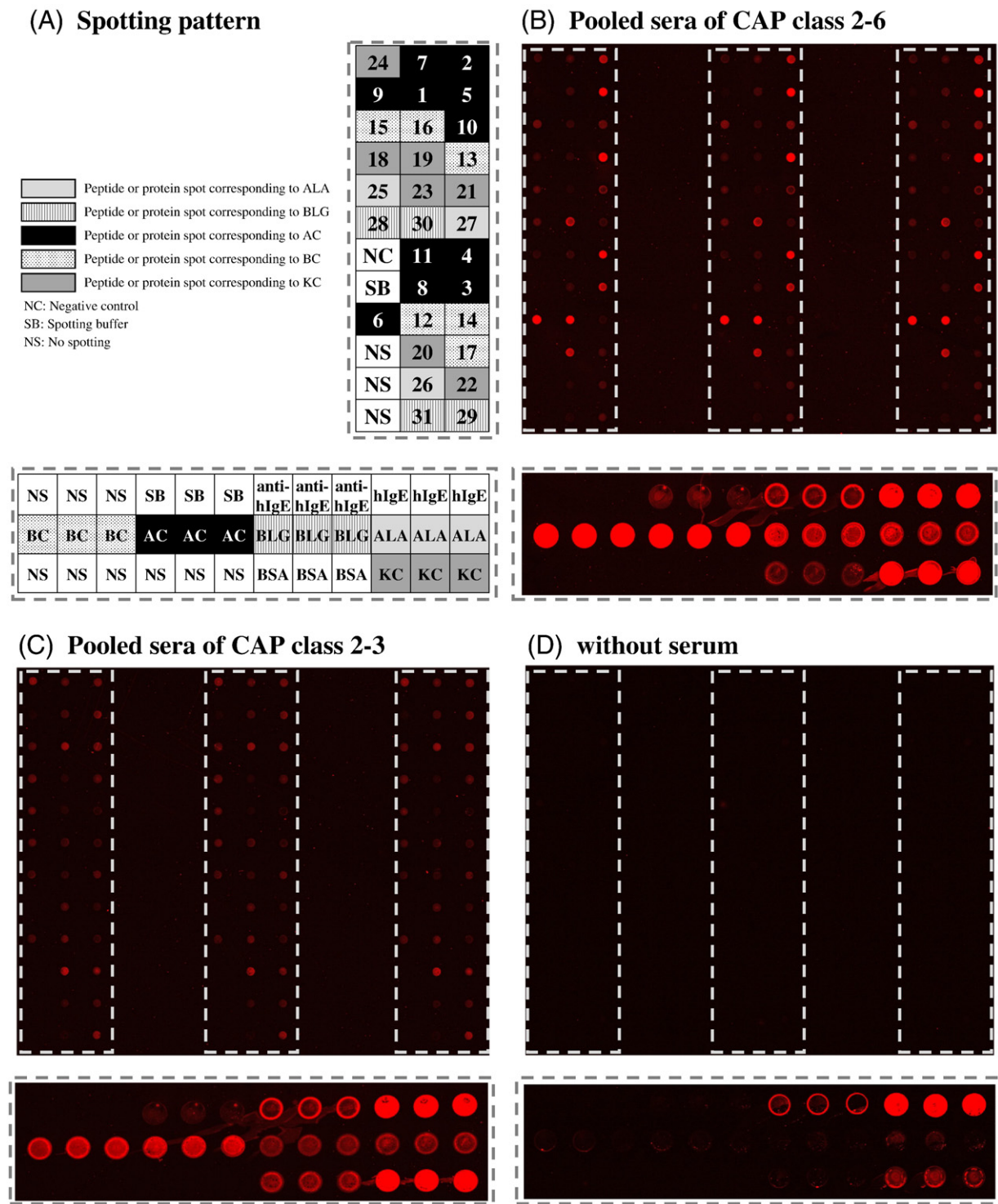


FIG. 1. The spotting test for FITC-labeled peptides using the piezoelectric micropump. FITC-labeled peptides were spotted using 20 mM phosphate buffer (pH 8.5) containing 0.1% SDS using the ceramic micropump. The fluorescence intensity (A) and mean spot diameter (B) with 95 spots were evaluated in order to assess the spotting accuracy. The CV of the fluorescent intensity and spot diameter were under 6% and 2%, respectively.



aqueous solution. Based on viewpoints such as ensuring uniformity in the spot diameter and automatic loading into the head of the micropump, we optimized a peptide dissolving solution. For peptide dissolving and peptide array development, we selected the 20 mM phosphate buffer (pH 8.5), containing 0.1% SDS. The peptide spotting test was performed using two FITC-labeled peptides to evaluate the stability and shape of the depicted spots using the ceramic micropump. The mean coefficient variation (CV) of the fluorescent intensity (median) and spot diameter were under 6 and 2%, respectively (Fig. 1). Therefore, it was shown that consistent spotting of peptides is possible using this method. Linear peptide array that contained linear peptide fragments in Table 1 which was reported as IgE-binding epitopes for cow's milk allergy (11–15) was constructed using this spotting method.

Detection of IgE-binding sites using pooled sera from CMA patients We examined two pooled sera from CMA patients using a peptide array that contained peptides in Table 1 (11–15) in order to detect the IgE-binding sites. Figs. 2B, 2C, and 2D show the fluorescence images analyzed using pooled sera from CMA patients with a CAP class 2–6, class 2–3, and without serum, respectively. The peptide array

consisted of triplicate spots of each peptide. When the spots with fluorescent intensities above the lower limit of detection ($S/N=2$) were analyzed, their mean CV was within 7% of both pooled sera from CAP class 2–6 and class 2–3. When we compared the fluorescent images, stronger fluorescence intensities were observed with the pooled sera of CAP class 2–6 than with those of class 2–3, as shown in Figs. 2B and C, respectively. The strong bindings to AC, BC, and KC were observed with the class 2–6 pooled sera, showing more than 100 kU/l in the CAP test for casein. Some of the peptide spots corresponding to these proteins also showed strong fluorescence intensity. Although non-specific bindings to anti-hIgE, ALA, and KC were slightly observed without serum, it was not observed with the peptides and the other proteins. Consequently, the obtained signals might be due to the specific binding of IgE in the sera. Therefore, it was confirmed that the developed peptide array enables the detection of the specific IgE recognition site, and it is possible to identify the IgE-binding epitopes in individual patients.

Identification of IgE-binding sites in CMA patient sera To evaluate the IgE-binding epitopes in individual patients, serum from

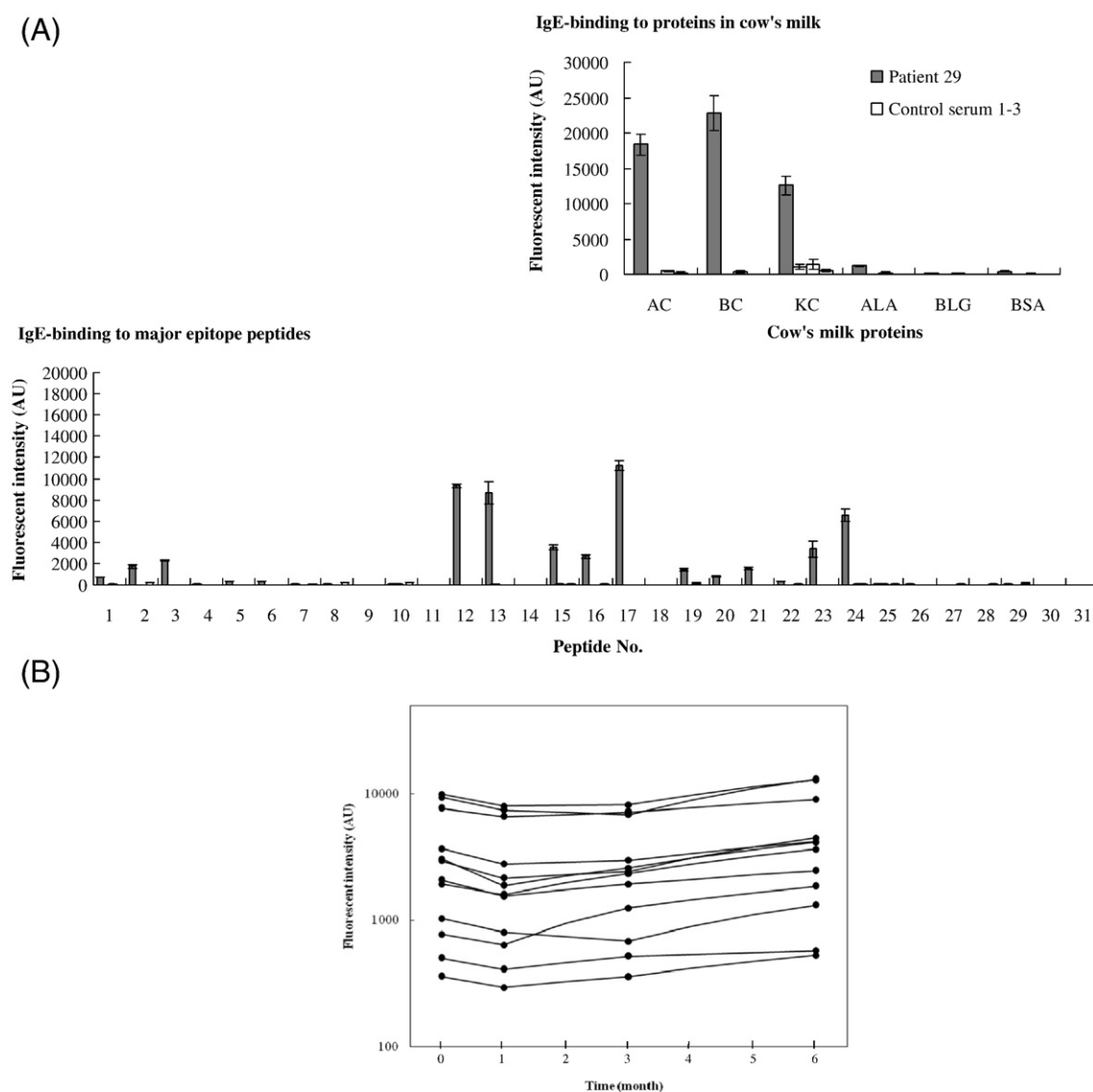


FIG. 3. Immunofluorescent assay using the CMA patient sera. (A) Detection of IgE-binding epitopes in the serum of CMA patient no. 29 and control sera nos. 1–3. The mean fluorescence values of the intra-assay for each peptide and protein are represented. (B) The stability of the synthesized peptide array via the piezoelectric micropump was analyzed using serum from CMA patient no. 29. Each line plot represents the temporal transition of the mean inter-assay fluorescence intensity (logarithmic scale) for each peptide with $S/N > 2$.

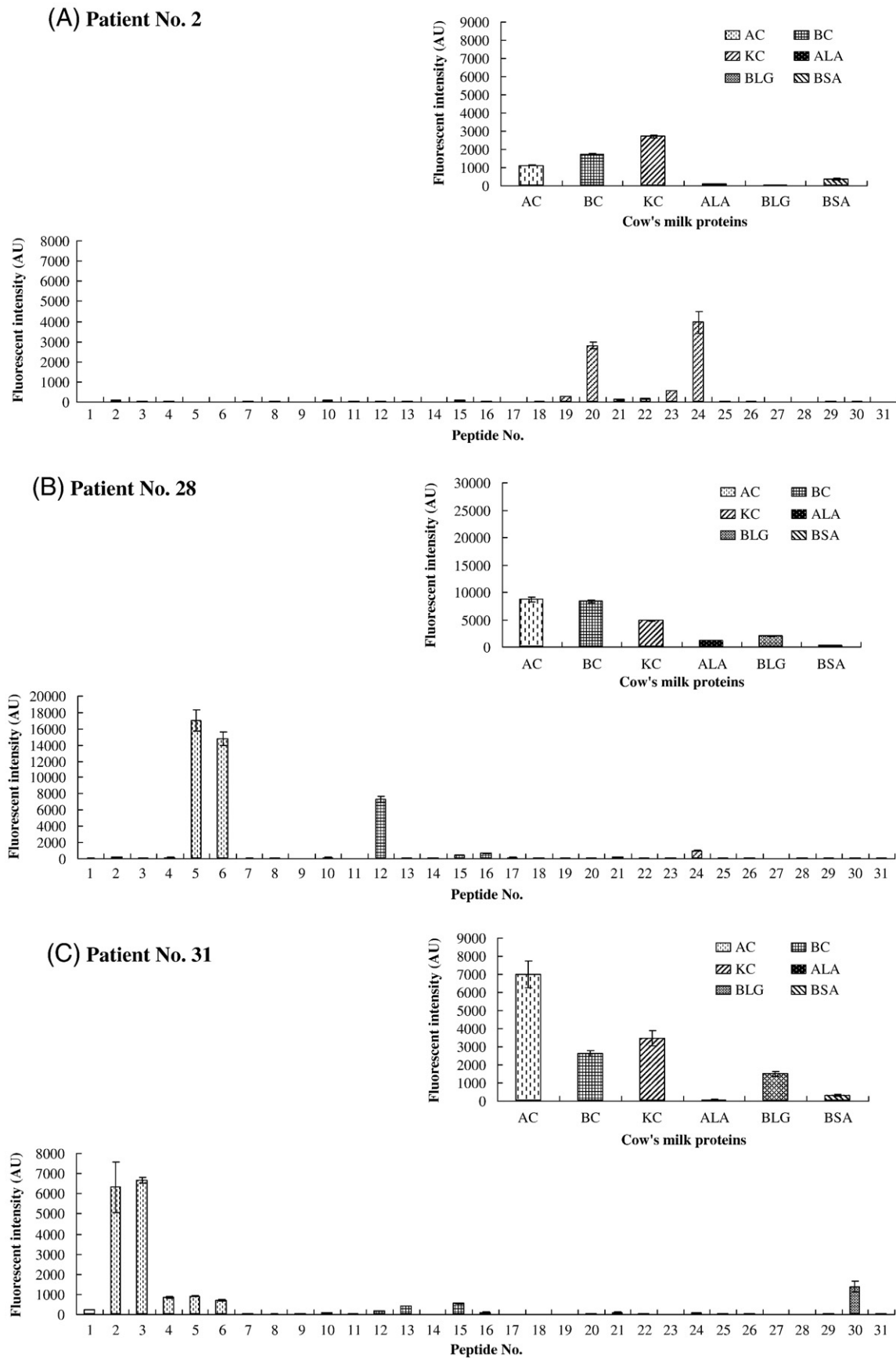


FIG. 4. Identification of IgE-binding to peptides, or proteins, using three patients with CMA. To analyze IgE reactivity to spotted peptides, we assayed the sera from three CMA patients: (A) patient no. 2, (B) patient no. 28, and (C) patient no. 31. The mean fluorescence intensity of the intra-assay for each peptide and protein are represented. Using the CAP test, the cow's milk-specific IgE values are 40.5, 82.5, and >100 kU/l in patient nos. 2, 28, and 31, respectively.

CMA patient no. 29 was analyzed. Patient no. 29 has a history of anaphylaxis and had a cow's milk-specific IgE value higher than 100 kU/l in the CAP test. The assay was also performed with 3 control sera to exclude the possibility of non-specific binding by various compounds included in the serum. Fig. 3A shows the results for patient no. 29 and control serum nos. 1–3. IgE-binding was observed in some of the peptides, especially in peptide nos. 12, 13, 17, and 24. In contrast, little binding was observed for the control sera with these peptides. Therefore, specific binding of patient serum IgE was observed using this peptide array. At the protein level, non-specific binding to KC was slightly observed in the control sera. Since KC binding was also observed with no serum (Fig. 2D), this might be due to the non-specific binding of a secondary antibody. In accordance with the protein data, the peptide epitopes corresponding to AC (peptide nos. 1–3, 5, and 6), BC (peptide nos. 12, 13, and 15–17), and KC (peptide nos. 19–24) were identified. Therefore, it was determined that IgE epitope identification is possible using our peptide array.

Stability of the peptide array The stability of peptide arrays was investigated. Peptide arrays were preserved in desiccators at room temperature for 1, 3, and 6 months and the change in the fluorescence intensities of each peptide with $S/N > 2$ was observed using 3 peptide arrays with triplicate spots from CMA patient no. 29's sera. When heat fixation was performed on the day of the immunoassay experiments, little change in the fluorescence intensities of each peptide was observed for over a 6 month period (Fig. 3B). In contrast, when the heat fixation treatment was performed before the peptide arrays were stored; the fluorescence intensities of each peptide varied, and increased the inter-array standard deviations (data not shown). Therefore, it was suggested that heat fixation of the peptide array should be performed after storage, as well as when preparing DNA microarrays (18), and the developed peptide array enabled the analysis of IgE-epitopes for at least 6 months without changing the quality of the analysis.

IgE-binding analysis with sera from CMA patients We assayed the recognition patterns of three CMA patients (Fig. 4). Patient no. 2 was mainly sensitized to casein (milk CAP 40.5 kU/l, casein CAP 68.6 kU/l, α -lactalbumin CAP < 0.35 kU/l, and β -lactoglobulin CAP < 0.35 kU/l). Patient nos. 28 and 31 exhibited milk CAP > 100 kU/l but without detailed CAP data on milk proteins. The assay result from patient no. 2 showed an IgE-binding to protein, AC, BC, and KC; and little binding to ALA and BLG was observed. Therefore, we obtained a comparable result for the IgE-binding to milk proteins using the CAP test. With respect to the peptide results, peptides nos. 20 and 24, derived from KC, showed a fluorescent intensity higher than 2000. There was little binding to peptides, other than the KC-derived peptides. Although only a limited number of peptides were spotted in this study, there were no dominant epitopes for AC and BC in this array. It was determined that the results of the peptide array enabled the identification of IgE-epitopes and we obtained comparable assay results in the protein spots using the CAP test.

A high protein binding to AC and BC was observed in the serum from patient no. 28, followed by binding to KC, and BLG. The result from patient no. 31's serum showed binding to AC, KC, BC, and BLG. Little antigenicity to BSA was observed with all three sera. These samples also showed IgE-binding to peptides corresponding to proteins that showed antigenicity. A strong IgE-binding to peptides nos. 5, 6, and 12 was observed in the serum from patient no. 28. Concerning the serum from patient no. 31, peptide nos. 2 and 3 exhibited a higher IgE-binding. The IgE-recognition peptide pattern varied among these serum samples, even if we only focus on peptide data corresponding to AC. These results indicate that the IgE-binding pattern might differ with individual patients, and the developed peptide array would be suitable for the analysis of IgE-binding epitopes in CMA patients.

DISCUSSION

Peptide arrays are an emerging technology in biomedical and pharmaceutical research and diagnosis. In the development of a peptide array, the use of a ceramic micropump enables the controlled spotting of peptides at a picoliter level, which is adapted for the miniaturization and mass production of the peptide array. Since peptides are formed from a combination of 20 amino acids, peptides properties, such as hydrophobicity (GRAVY (Grand Average of Hydropathy), the GRAVY value for a peptide is calculated as the sum of hydropathy values (19) of all the amino acids, divided by the number of residues in the sequence), vary widely. Thus, it is difficult to dissolve peptides uniformly in an aqueous spotting solution and to ensure consistent spotting. To improve this problem, we used a solution containing the detergent, SDS, in the spotting buffer to prepare the peptide solutions. The use of detergent in the spotting buffer reduces the buffer's surface tension and allows the immobilization of peptides with a small CV for the immobilization amounts and spot diameters values (Fig. 1). The peptide spotting technique developed in this study can be used to spot all of the peptides with overlapping milk protein sequences as well as a diagnostic tool for epitope mapping.

Peptide arrays have the potent capability to identify immunodominant epitopes of allergen proteins, and provide useful information to understand the IgE epitopes responsible for allergic reaction. In the current study, a conventional immunofluorescent assay was successfully performed to measure allergen-specific IgE (Figs. 2–4). For food allergens, it is important to identify the linear epitopes since allergen proteins are fragmented by digestive enzymes and gastric acidity after ingestion. It is suggested that protracted food hypersensitivity is associated with the development of significant quantities of IgE antibodies against linear epitopes (16, 20). It is interesting to note that, the IgE recognition patterns for casein-derived peptides are different in the serum from each individual patient. These patterns might be associated with the onset of different responses to ingestion, site of origin or severity of symptoms. Also, binding of IgE to milk protein derived peptides (Table 1) was not observed in the control sera (Fig. 3A). Therefore, it is possible to use the peptide array as a diagnostic tool for identification of clinically meaningful epitopes. It is also possible for identification of IgG-binding epitopes that might compete with the binding of serum IgE to the specific epitope peptides. Detection of the competing interaction of IgE and IgG-binding would be attractive and useful for diagnosis. Although CAP-RAST has been widely used in diagnoses, the correlation between the IgE level of CAP and the severity of the allergen reaction is sometimes not observed (7). The development of a peptide array with clinically meaningful epitope peptides would be important to construct a diagnostic tool that minimizes the problems with false positive and negative results in the blood tests that are currently used.

The use of a peptide array for the analysis of IgE-binding epitopes in allergy diagnosis will provide accurate information and it will eliminate the risk of oral food challenges. Further studies are necessary to develop a peptide array that can be used as a diagnostic tool in clinical fields.

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