

Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy

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Background: Results from large-scale epitope mapping with a peptide microarray have been shown to correlate with clinical features of milk allergy.

Objectives: We sought to assess IgE and IgG4 epitope diversity and IgE affinity in different clinical phenotypes of milk allergy and identify informative epitopes that might be predictive of clinical outcomes of milk allergy.

Methods: Forty-one subjects were recruited from a larger study on the effects of ingesting heat-denatured milk proteins in subjects with milk allergy. Using food challenges, subjects were characterized as being clinically reactive to all forms of milk ($n = 17$), being tolerant to heated milk (HM) products ($n = 16$), or having outgrown their milk allergy ($n = 8$). Eleven healthy volunteers without milk allergy served as control subjects. A peptide microarray was performed by using the previously published protocol.

Results: Subjects with milk allergy had increased epitope diversity compared with those who outgrew their allergy. HM-tolerant subjects had IgE-binding patterns similar to those who had outgrown their allergy, but IgG4-binding patterns that were more similar to those of the allergic group. Binding to higher numbers of IgE peptides was associated with more severe allergic reactions during challenge. There was no association between IgG4 peptides and clinical features of milk allergy. Using a competitive peptide microarray assay, allergic patients demonstrated a combination of high- and low-affinity IgE binding, whereas HM-tolerant subjects and those who had outgrown their milk allergy had primarily low-affinity binding.

Conclusions: Greater IgE epitope diversity and higher affinity, as determined by using the peptide microarray, were associated with clinical phenotypes and severity of milk allergy. (*J Allergy Clin Immunol* 2010;125:695-702.)

Key words: Milk allergy, peptide microarray, IgE epitope, IgE affinity, IgG4 epitope

Cow's milk hypersensitivity is a common disease affecting 2.5% of infants in the first year of life,¹ with approximately 60% of these milk disorders caused by IgE-mediated mechanisms. Although tolerance develops in the majority of children with IgE-mediated milk allergy by their teenage years,² 15% to 20% have lifelong allergy. The mechanisms responsible for tolerance are still not clearly understood; it has been shown that the presence of IgE antibodies to distinct allergenic epitopes of cow's milk proteins can be used as a marker of persistent allergy.³ Furthermore, a recent study demonstrated that the majority of children with milk allergy can tolerate extensively heated forms of milk, and this subset of patients with milk allergy are more likely to become tolerant to milk over time.⁴

The current diagnostic modalities for food allergy include skin prick testing and measurement of serum specific IgE levels. These results give an indication of the likelihood of clinical reactivity; however, individual results do not provide prognostic information or distinguish between the different phenotypes of food allergy. The importance of sequential epitope recognition in the persistence of cow's milk allergy has been highlighted in several studies using SPOTS membrane technology.⁵⁻⁷ However, this method is time and labor intensive. Recently, peptide microarrays have been developed for large-scale epitope mapping with small quantities of serum.⁸ Shreffler et al⁹ used the peptide microarray immunoassay to examine serum samples from patients with peanut allergy and confirmed that antigenic areas identified by using this method correlated with areas defined by means of SPOTS membrane mapping. Furthermore, epitope recognition correlated with peanut allergy severity.^{9,10} Similarly, peptide microarray results have been shown to correlate with clinical features of milk allergy. Patients with milk allergy and milk-tolerant patients demonstrated different epitope recognition patterns, with allergic patients having higher ratios of IgE to IgG4 binding than those tolerant to milk.¹¹ Decreases in IgE binding and increases in IgG4 binding to milk peptides were correlated with clinical improvement in children undergoing oral immunotherapy with milk.¹² Differences in epitope diversity appear to be associated with clinical features of food allergy. Studies of milk allergenic epitopes have further demonstrated that certain milk IgE epitopes can be used as candidate biomarkers to predict the development of tolerance to milk.

In addition to epitope diversity and the concentration of epitope-specific antibodies, IgE antibody affinity (the binding

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Abbreviations used

HM: Heated milk
HSA: Human serum albumin
PBS-T: PBS containing 0.05% Tween 20

strength between antibodies and allergen or allergenic epitopes) might also be involved in the pathogenesis of allergic diseases. In this study we sought to determine whether IgE and IgG4 epitope diversity and affinity of IgE antibodies were correlated with the different clinical phenotypes for milk allergy, including patients with milk allergy, milk-tolerant patients, and the intermediate group of patients with milk allergy who can tolerate extensively heated forms of milk (ie, baked-milk tolerant), and to identify informative epitopes that might be useful in predicting the clinical outcome of milk allergy.

METHODS**Patient sera**

Sera previously used for mapping milk protein epitopes by using SPOTS membrane technology³ were used to confirm antigenic areas identified by means of peptide microarray.

Forty-one subjects were recruited from a larger clinical study on the effects of ingesting heat-denatured milk proteins in subjects with milk allergy to assess the association between epitope diversity and the clinical phenotype of milk allergy. Subjects were characterized based on the results of food challenges as allergic (ie, reactive to all forms of milk products; $n = 17$), heated cow's milk tolerant ($n = 16$), or having outgrown ($n = 8$) their milk allergy. The HM-tolerant group all tolerated heated milk (HM) in the form of muffins, waffles, and pizza but reacted to regular cow's milk. Blood samples from subjects were obtained at the time of the initial baseline challenge. Eleven healthy volunteers without milk allergy served as control subjects. All research protocols were approved by the Mount Sinai Institutional Review Board, and informed consent was obtained for all subjects. Allergen-specific IgE levels and skin test data were obtained, as previously described.⁴

Microarray

Peptides, slides, printing. A library of peptides, consisting of 20 amino acids overlapping by 17 (3-offset) and corresponding to the primary sequences of α_{s1} -, α_{s2} -, β -, and κ -caseins and β -lactoglobulin, was commercially synthesized. Peptides were resuspended in dimethyl sulfoxide at 2 mg/mL, diluted 1:2 in Protein Printing Buffer (TeleChem International, Inc, Sunnyvale, Calif) with 0.02% Sarkosyl to a final concentration of 1 mg/mL, and printed on epoxy-derivatized glass slides (SuperEpoxy Substrate; TeleChem International, Inc) by using the NanoPrint Microarrayer 60 (TeleChem International, Inc). Protein Printing Buffer alone was used as a negative control and for background normalization, and fluorochrome-labeled BSA used for the purpose of grid alignment during analysis was included on each slide. All array elements were printed in duplicate (2 sets of triplicates) to improve precision and determine intra-assay variation.

Immunolabeling. Immunolabeling was performed as previously described, with some modifications.^{12,13} In brief, the slides were blocked with 400 μ L of 1% human serum albumin (HSA) in PBS containing 0.05% Tween 20 (PBS-T) for 60 minutes at room temperature, followed by incubation with 250 μ L of patient serum diluted 1:5 in PBS-T/HSA for 24 hours at 4°C. Slides were then washed with PBS-T and incubated for 24 hours at 4°C with a cocktail of several mAbs, including 3 biotinylated monoclonal anti-human IgE antibodies, one from Invitrogen (Carlsbad, Calif) diluted 1:250, one from BD Biosciences Pharmingen (San Jose, Calif) diluted 1:250, and one as a gift from Phadia (Uppsala, Sweden) biotinylated in our laboratory and diluted 1:1000, and one monoclonal anti-human IgG4-fluorescein isothiocyanate (SouthernBiotech, Birmingham, Ala) diluted 1:1000 in PBS-T/HSA. Slides

TABLE I. Patient characteristics

	Outgrown group (n = 8)	HM-tolerant group (n = 16)	Allergic group (n = 17)
Age (y)			
Median	5.8	5.7	7.3
Mean	7.9	6.6	7.7
Range	2.61–14.69	4.84–11.36	3.43–14.91
Interquartile range (25% to 75%)	3.38–12.94	5.49–6.95	4.66–9.77
Sex (F/M)	4/4	4/12	9/8
Milk IgE (kU/L)			
Median	0.9*	1.8*	11.6*
Mean	1.4	3.23	27.7
Range	0.36–3.23	0.35–14.7	0.77–>100
Interquartile range (25% to 75%)	0.41–2.45	0.75–3.7	5.97–37.4

*Statistically significant difference in milk IgE levels between the allergic and HM-tolerant groups ($P = .004$) and between the allergic and outgrown groups ($P = .02$).

TABLE II. Number of peptides bound by IgE and IgG4

	Outgrown group	HM-tolerant group	Allergic group
No. of peptides bound by IgE			
Mean	12.3	14.6	46.6
Median	3.5	3*	17*
Range	0–66	0–103	0–186
No. of peptides bound by IgG4			
Mean	19	9.1	14.7
Median	9	6.5	13
Range	0–93	0–40	0–56

There was a significant difference between allergic and HM-tolerant subjects. There were no differences in IgG4 binding between groups. Peptide is positive if z score is greater than 3.

*Statistically different number of peptides bound by IgE between the HM-tolerant and allergic groups ($P = .019$).

were then incubated for 3 hours at 31°C with a cocktail of Anti-Biotin Dendrimer Oyster 550 (350; Genisphere, Hatfield, Pa) and Anti-Fluorescein Isothiocyanate Dendrimer Oyster 650 (350; Genisphere) in Dendrimer Buffer (Genisphere), both at 0.6 μ g/mL, with the addition of 0.02 μ g/mL salmon sperm DNA (Invitrogen), followed by washing with PBS-T, 15 mmol/L Tris, 0.1× PBS, and 0.05× PBS. Slides were centrifuge dried and scanned with a ScanArrayGx (PerkinElmer, Waltham, Mass). Images were saved in TIF format.

Competition assay. The sera from 7 subjects with milk allergy and 7 HM-tolerant/outgrown subjects with binding to multiple milk peptides, with the majority of binding showing z scores of greater than 5, were selected for a competition assay. The competition assay was performed by immunolabeling the slide as described above with an additional incubation with a mixture of 1 mg/mL casein (Sigma-Aldrich, St Louis, Mo) and 1.6 mg/mL β -lactoglobulin (Sigma-Aldrich) at 16°C for 1 hour after serum incubation and before application of the second antibody.

Data and statistical analysis

The fluorescence signal of each spot was digitized with the program ScanArray Express (PerkinElmer), exported as comma-delimited text files and transformed to a z score, as previously described.^{11,12} An index z value of each peptide element was generated from the median of z scores of the 6 replicate spots. An individual peptide sample was considered positive if its z score exceeded 3, meaning that the signal was significantly above the background ($P < .003$). Single-positive peptides with negative binding by 4 adjacent peptides were filtered out by means of replacement with the median

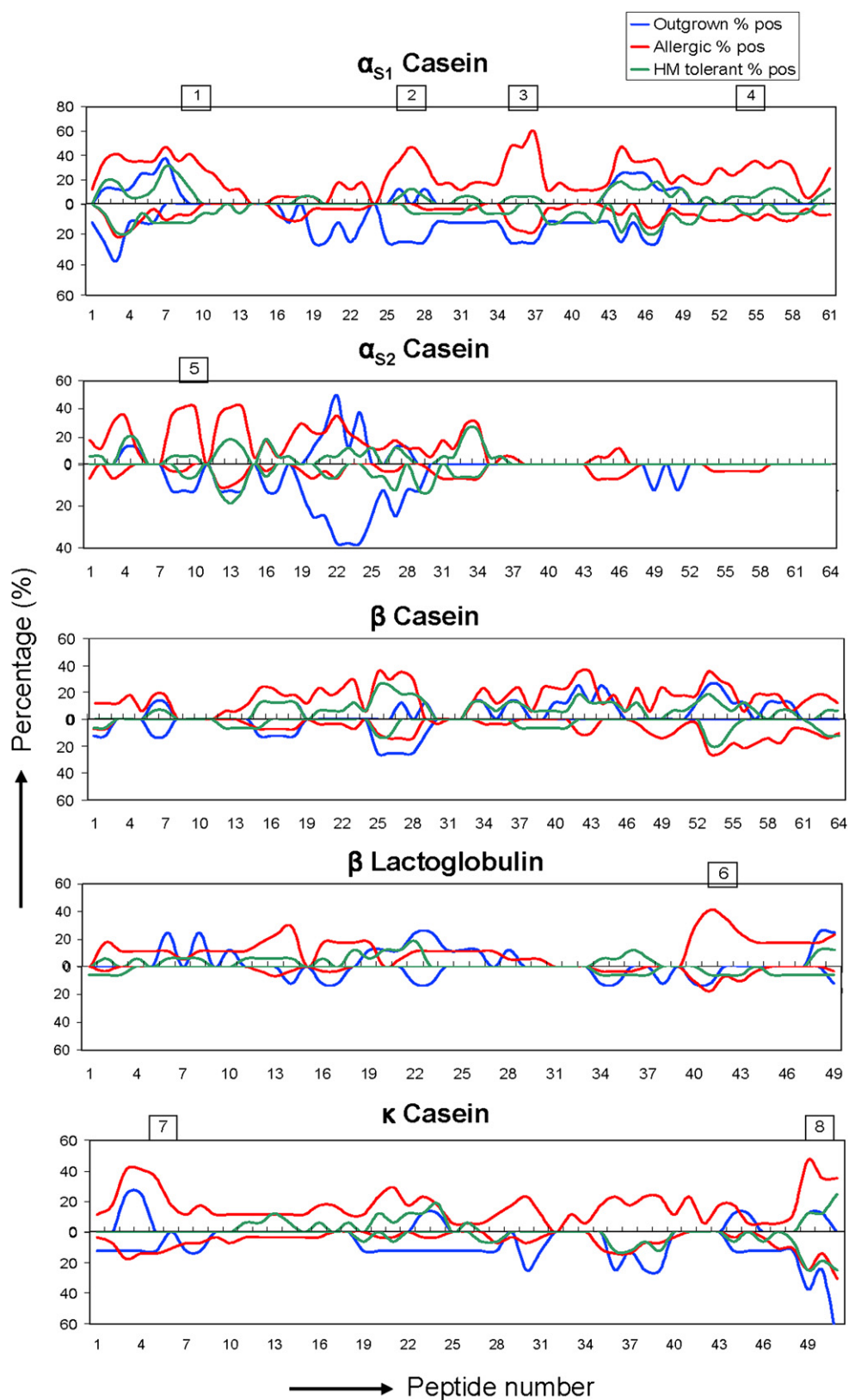


FIG 1. Comparison of IgE and IgG4 binding frequency to peptides of 5 milk allergens between the different clinical phenotypes. The *x-axis* shows the overlapping peptides, and the *y-axis* shows the percentage of patients within each group showing positive binding to each peptide. Binding for IgE is indicated above the *x-axis* line; binding for IgG4 is below the line. Candidates of informative epitopes are indicated with numbers.

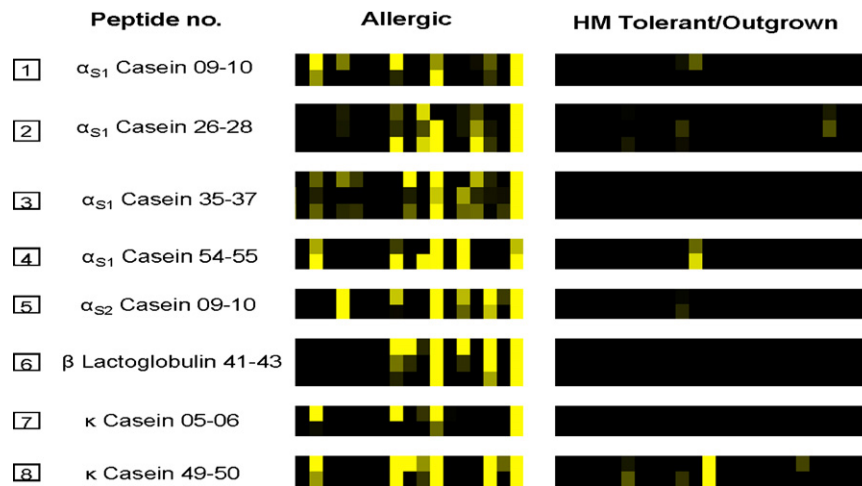


FIG 2. Heat map of IgE binding to candidates of informative epitopes from the allergic subjects compared with the HM-tolerant/outgrown subjects.

of itself and the 4 adjacent peptides to eliminate nonspecific background noise.

To identify candidates of informative epitopes that would distinguish between the clinical phenotypes, we used the following procedure. The Wilcoxon test was used to determine statistical differences in reaction levels (signal intensity) to each peptide between groups. If 2 or more adjacent peptides demonstrated significantly different binding ($P < .05$) between groups, the amino acids spanning these peptides were considered a possible informative epitope.

The binding frequency of each group, which is the percentage of patients who had a z score of greater than 3 at each peptide, was also calculated. For identification of informative epitopes, only areas showing a greater than 30% difference in binding frequency between groups were considered.

Informative epitopes were identified as peptides that showed significantly different binding intensities between groups by using the Wilcoxon test and greater than 30% difference in binding frequency. Additional informative epitopes were identified based on IgE binding affinity. Amino acids of the informative epitopes were mapped based on the overlapping sequences of the identified peptides. Data presentation was performed with Microsoft Excel and the TIGR Multiexperiment Viewer (Dana-Farber Cancer Institute, Boston, Mass).

RESULTS

Antigenic areas identified with the microarray correspond well to previously identified regions

Using the serum samples previously used for SPOTS membrane mapping, we confirmed that allergenic/antigenic areas identified with the peptide microarray corresponded well with previously identified regions. Furthermore, the microarray was able to detect binding in some areas where binding was undetectable by means of SPOTS membrane mapping, indicating that the microarray might be more sensitive than SPOTS membrane technology (data not shown).

IgE diversity corresponds to different phenotypes of milk allergy

Characteristics of the patient samples from the clinical study on the natural history of milk allergy are listed in Table I. IgE from patients who have outgrown their milk allergy tended to bind fewer milk peptides compared with IgE from those with persistent

milk allergy (ie, allergic patients; median number of peptides bound by IgE, 3.5 vs 17; $P = .062$), but there was no difference in IgG4 binding of peptides. IgE from HM-tolerant subjects bound significantly fewer IgE peptides than that from the allergic group (median IgE peptides bound, 3 vs 17; $P = .019$) and bound fewer peptides with IgG4 than either those who had outgrown their milk allergy or those who were still allergic (not statistically significant; Table II and see Figs E1 and E2 in this article's Online Repository at www.jacionline.org).

IgE and IgG4 binding frequencies to each peptide by group are shown in Fig 1. Subjects who were HM tolerant had IgE-binding patterns similar to those of subjects who had outgrown their milk allergy but IgG4-binding patterns that were more similar to those of the allergic group.

IgE binding frequency was correlated with severity of symptoms during oral food challenge. Children in the allergic group who had a higher reaction grade during the challenge (anaphylaxis grade 4-5) bound a median of 89.5 IgE-binding peptides, whereas children who reacted with anaphylaxis grades 1 to 2 bound a median of 4.5 IgE-binding peptides ($P = .02$). The HM-tolerant group generally had milder reactions during their oral food challenges to milk (grades 1-2 only) and bound a median of 3 IgE-binding peptides. There was no correlation between the number of IgG4 peptides and the severity of allergic reactions during challenge or between the number of IgE or IgG4 peptides bound and the eliciting dose during challenge.

HM-tolerant subjects and those who had outgrown their allergy were combined as one group (HM-tolerant/outgrown group, $n = 24$) and compared with the allergic subjects ($n = 17$) to identify candidates of IgE or IgG4 informative epitopes. Only areas showing a greater than 30% difference in binding frequency between groups were considered. Candidates of informative epitopes were identified as described in the Methods section.

Eight areas that fulfilled the criteria listed in the Methods section were identified as informative epitopes for IgE (Figs 1 and 2). The majority were located in α_{s1} -casein, correlating with SPOTS-identified epitopes.⁵⁻⁷ The corresponding IgE epitopes were identified on the protein sequence in Fig 3. There were no areas with a greater than 30% difference between groups for IgG4.

α_{s1} Casein
 RPKHPKIHQGLPQEVLENENLLRFFVAP**FPEVFGKEKVNELSKDI**GSESTE
 DQAMEDIKQMEAESSISSEIIVPNSVE**QKHIQKEDVP**SERYLGYLEQLLR
 LKKYKVPQ**LEIVPNSAEERLHSM**KEGIHAQQKEPMIGVNQELAYFYPELF
 RQFYQLDAYPSG**AWYYVPLGTQYTDAPSF**SDIPNPIGSENSEKTTMPLW

α_{s2} Casein
 KNTMEHVSSSEESIISQETYSQEKQKNMA**INPSKENLCSTFC**KEVVRNANEE
 EY**SI**IGSSSEESAEEVATEEVKITVDDKHQKALNEINQFYQKFPQYLQYLY
 QGPVLNPNWDQVKNRAVPITPTLNREQLSTSEENSKKTVDMESTEVFTKK
 TKLTEEKNRLNFKKISQRYQKFALPQYLKTVYQHQAAMKPWIPKTKV
 IPYVRYL

β Casein
 RELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQTDELQDKIH
 PFAQTQSLVYPPGPIPNLQNIPLT**QTPVVVPFLOPEVMG**VSKVKE
 AMAPKHKEMFPKYPVEPFTESQSLTLTDVENLHLLPLQLQSWMHQHPQ
 LPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGP
 VRGPFPIIV

β Lactoglobulin
 LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTP
 EGDLEILLQKWENDECAQKKIIAEKTKIPAVE**KIDALNENKVL**LDTDYK
 KYLLVCMENSAEPEQSLVCQCLVRT**EVDDALEKFDKAL**KALPMHIRLS
 FNPTQLEEQCHI

κ Casein
 QEQNQEOPIRCEKDE**RFFSDKI**AKY**PIPIQYVLS**RYPSYGLNYYQQKPVAL
 INNQFLPYPYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPH
 PHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLE**DSP**
EVIESPPPEINTVQVTSTAV

FIG 3. Amino acid sequence of milk allergens. Candidates of informative epitopes identified based on the binding frequency are in bold and underlined. Informative epitopes recognized by greater than 50% of the allergic patients with high-affinity IgE antibodies are highlighted in gray. The numbers and letters indicated on top of each epitope correspond to the ones indicated in the other figures.

Sera from subjects without milk allergy were used as controls. Eight of the 11 sera had no detectable IgE binding, and 3 control subjects had IgE that bound a median of 9 milk peptides. The majority had IgG4 binding to milk peptides (median, 2.5 peptides).

Of note, within each clinical group, there was heterogeneity in terms of IgE and IgG4 binding to milk peptides, with each subject having a unique pattern of binding.

IgE affinity correlates to different phenotypes of milk allergy

A competition assay was performed with a subset of patients who had binding to multiple milk epitopes to determine the relationship between IgE antibody affinity and clinical allergy phenotype, with the majority of binding showing z scores of greater than 5 (7 allergic subjects, 5 HM-tolerant subjects, and

2 subjects who had outgrown their allergy). The competition conditions, including the concentration of competitor proteins, temperature, and length of incubation, were first determined by using a serum pool of 5 subjects highly allergic to milk (see Figs E3-E5 in this article's Online Repository at www.jacionline.org). No competition effect could be observed from the replicate arrays incubated with PBS-T/HSA (data not shown).

Compared with the standard protocol, the competition assay using the caseins and β -lactoglobulin virtually eliminated all IgE binding from the HM-tolerant/outgrown group (Fig 4). No competition effect was observed in arrays incubated with PBS-T/HSA. For patients in the allergic group, IgE binding was affected to a much lesser degree (Fig 5). For allergic patients, IgE antibodies to certain epitopes remained bound (z score > 3) after competition, and this z score decreased less than 50% compared with the standard protocol, suggesting that they are high-affinity antibodies. In general, all allergic patients had a mixture of high- and

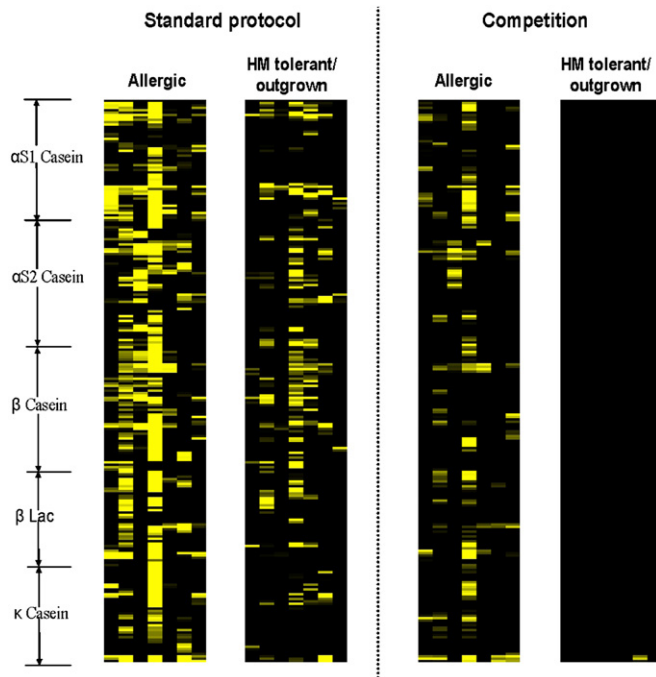


FIG 4. Heat map showing the effects of competition on 7 allergic and 7 HM-tolerant/outgrown subjects.

low-affinity antibodies to different epitopes. There were 3 epitopes (A-C; Fig 6) recognized by more than 50% of the allergic patients whose specific IgE antibodies to these epitopes were of relatively high affinity. One epitope overlapped with the informative epitopes identified with the standard protocol (C in Fig 3). Two additional informative epitopes (A and B) with high antibody affinity are also indicated in Fig 3.

DISCUSSION

The aim of this study was to determine whether IgE and IgG4 milk allergen epitope recognition and IgE antibody affinity correlated with different phenotypes of milk allergy. First, we confirmed that antigenic areas identified with the peptide microarray correspond well to regions previously identified with SPOTS membrane technology by using stored serum from subjects participating in the original study.³ Next, we demonstrated that IgE epitope diversity on the peptide microarray correlated with clinical phenotypes of milk allergy. Patients with persistent allergy had increased epitope diversity compared with those with transient allergy (outgrown). Immunologic studies from the HM study suggest that those who are HM tolerant have a favorable prognosis and are less clinically reactive than those who are unable to tolerate HM.^{4,14,15} Our microarray data are consistent with these results because IgE from HM-tolerant subjects recognized fewer milk allergen peptides from those who were unable to tolerate HM and were more similar to those who had outgrown their allergy. Furthermore, binding to increased numbers of IgE peptides correlated with the severity of allergic reactions during oral food challenge.

IgG4 binding to milk peptides was detected in the majority of patients in all groups. The HM-tolerant group tended to have the least amount of IgG4 binding to milk peptides. This was similar to the reported lower quantitative IgG4 levels to casein and

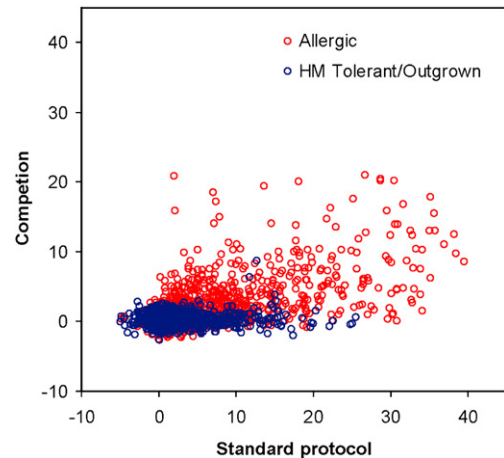


FIG 5. Scatter plot comparing IgE binding from 7 allergic (red) and 7 HM-tolerant/outgrown (blue) subjects between the standard protocol (*x*-axis) and the competition assay (*y*-axis). Each spot represents IgE binding (represented as a *z* score) to 1 milk peptide.

β -lactoglobulin in the HM-tolerant group.⁴ Because strict avoidance is currently the mainstay of treatment, it is possible that IgG4 binding might increase as the HM-tolerant group incorporates certain HM products into their diets. Alternatively, as tolerance to milk develops, subjects might lose IgG4 to some epitopes and develop IgG4 to other areas of the milk proteins.

Although several IgE epitopes bound by a significantly higher percentage of allergic subjects were identified in our study, no single informative epitope that reliably distinguished between the different phenotypes of milk allergy could be identified because of the heterogeneity of epitopes recognized by subjects within the different phenotypes, which is similar to observations in studies of the peanut microarray.^{9,10} Previous studies have indicated that the number of epitopes recognized, rather than recognition of specific epitopes, might be more predictive of clinical features of food allergy.^{16,17} However, an overlap in the number of epitopes recognized was also observed.

A possible explanation for the heterogeneity of IgE binding within the allergic group is the relatively young age of the study population (median age, 7.3 years). A recent report indicated that some patients do not outgrow their milk allergy until their early teenage years.² Therefore it is possible that some of the subjects currently classified as allergic will eventually outgrow their milk allergy and thus should be reclassified in a different group.

Although epitope diversity to peanut allergens appears stable,¹⁰ epitope diversity for milk allergens can change over time. In a study of patients undergoing oral immunotherapy with milk, children who were successfully desensitized demonstrated decreased IgE binding and increased IgG4 binding after treatment.¹² The opposite trend was noted in those who were unable to be desensitized. An important difference between these 2 studies is the therapeutic intervention in the milk study as opposed to the peanut study, in which subjects continued peanut avoidance. Therefore it is possible that continued exposure to milk in the form of HM products in our patients will lead to immunologic effects on epitope-binding patterns similar to those seen in children undergoing oral immunotherapy. Alternatively, peanut allergy is generally more persistent, and therefore characteristics of epitope recognition might be different for different food proteins.

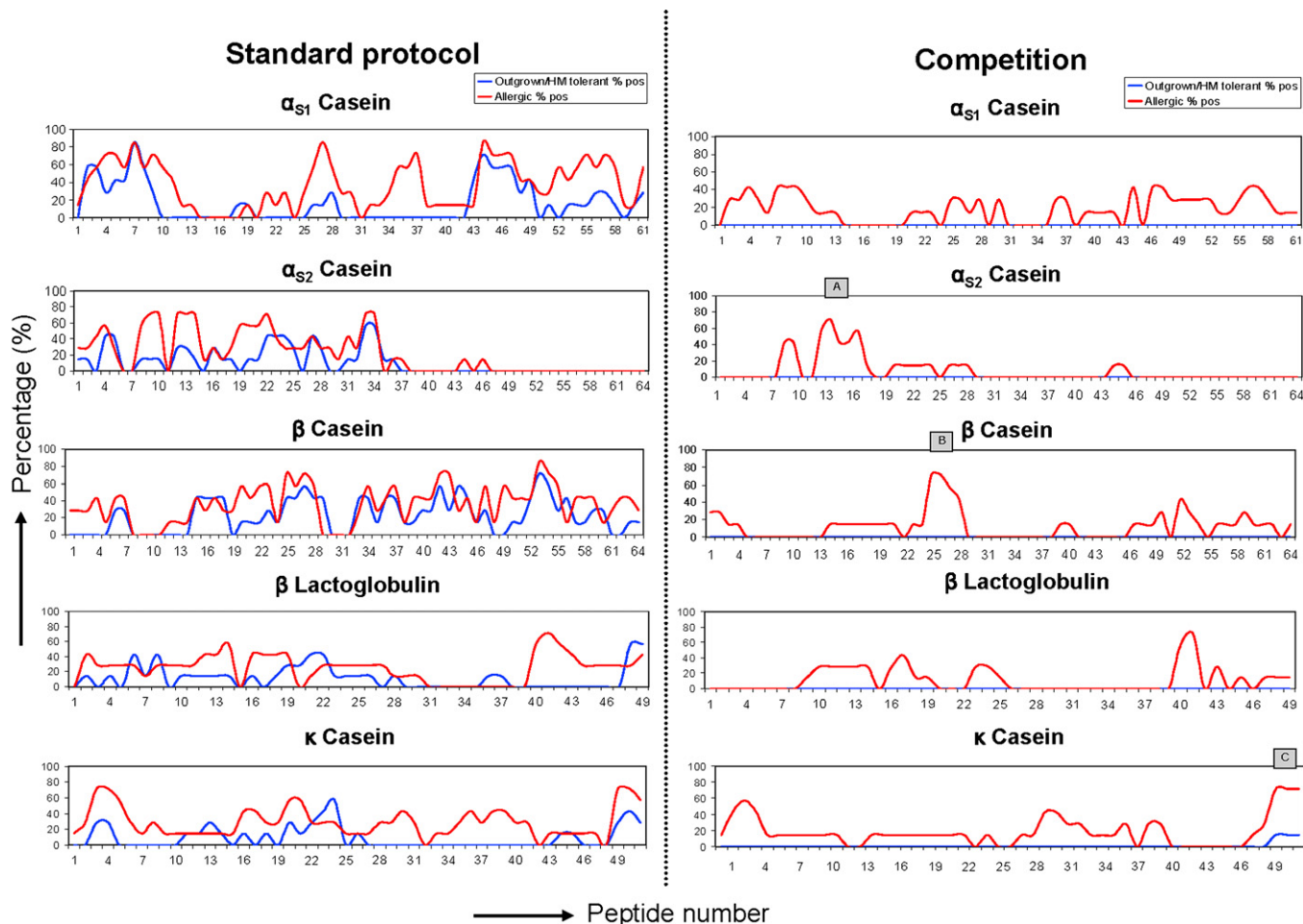


FIG 6. Comparison of IgE peptide-binding frequency between groups using the standard protocol and competition assay. Informative epitopes recognized by greater than 50% of the allergic patients with high-affinity IgE antibodies are indicated with letters.

A novel finding in our study is that IgE affinity is correlated with clinical features of milk allergy. This is the first time that antibody affinity has been shown to closely reflect clinical characteristics of milk allergy. Patients with milk “allergy” have IgE antibodies that bind to certain peptide segments of milk proteins with high affinity and that are not competed away by native milk protein. In contrast, those who have transient allergy (outgrown) or are HM tolerant have low-affinity binding that is completely competed away by native milk protein. These results suggest that IgE affinity might play a role in the development of tolerance as well.

Affinity maturation is an important step in the progressive differentiation of antigen-specific B cells, leading to antibody clones with higher affinity. Thus affinity might be one factor in determining the biological effects of IgE antibodies. However, the role of antibody affinity in allergy has not been well described. One early report investigated the effect of IgE antibody affinity to Der p 2 on Der p 2–induced histamine release from human basophils and found that the sensitivity of histamine release was closely related to the affinity.¹⁸ Recently, Christensen et al¹⁹ demonstrated that higher-affinity IgE binding to Der p 2 was associated with higher levels of basophil activation. There is one published report investigating antibody avidity in peanut allergy.

Using a thiocyanate ELISA elution assay, the authors found only a weak correlation between IgE and IgG avidity for Ara h 2 and symptom score during oral food challenge.²⁰ Thiocyanate elution works by nonspecifically dissociating antigen–antibody complexes by interfering with noncovalent binding. In contrast, native milk protein was used in our study as a competitive inhibitor for IgE binding sites, which might be a more relevant indicator of antibody affinity in patients with food allergy. Furthermore, when only antibodies with higher affinity were considered, the overlap observed in the epitope recognition pattern could be eliminated, resulting in a clearer distinction between groups.

Additional studies are necessary to confirm these findings in larger numbers of patients and to determine whether changes in epitope diversity and affinity occur over time as a result of therapeutic interventions, a part of the natural history of food allergy, or both. This information might provide additional insight into the mechanisms of tolerance. On the other hand, if epitope diversity and affinity are found to be stable over time, peptide microarrays might be a useful diagnostic tool that can provide prognostic information regarding development of tolerance and severity of milk allergy based on the affinity profile and epitope diversity, respectively. The current diagnostic tools are unable to provide this type of prognostic information, and therefore the

peptide microarray might represent a much needed advancement in diagnostics for the field of food allergy.

Clinical implications: IgE epitope diversity and affinity correlate with clinical phenotypes and the severity of milk allergy. The peptide microarray might be a useful diagnostic tool that can provide prognostic information regarding milk allergy.

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RESULTS

Optimization of the competition conditions

Effect of temperature. The effect of temperature on competition was tested by using a pool of sera from 5 patients highly allergic to milk and with milk-specific IgE levels of greater than 100 kUA/L. Three replicate arrays were immunolabeled with the serum pool and competed with 2 mg/mL caseins and 3 mg/mL β -lactoglobulin at different temperatures (31°C, 16°C, and 4°C) for 4 hours. One replicate array was processed according to the standard protocol as a control.

As shown in Fig E3, IgE binding from the serum pool, which contains a mixture of high- and low-affinity antibodies, was reduced to different degrees, possibly reflecting the antibody affinity. The competition effect increased as the temperature increased.

Effect of competitor concentration. The effect of competitor concentration was also tested by using the same serum pool. Three replicate arrays were immunolabeled with the serum pool and competed with a one-third serial dilution of 2 mg/mL caseins and 3 mg/mL β -lactoglobulin at 16°C for 4 hours. As shown in Fig E4, the competition effect decreased as the competitor concentration decreased. The competitor concentration was set at 1 mg/mL caseins and 1.5 mg/mL β -lactoglobulin.

Effect of competition time. The effect of competition time was tested with the sera of a subject with milk allergy and a subject who had outgrown his or her allergy by using 1 mg/mL caseins and 1.5 mg/mL β -lactoglobulin at 16°C for different competition times (Fig E5). One hour of competition was selected as the optimal competition time because the competition effects of the other 2 times were either too weak or too strong.

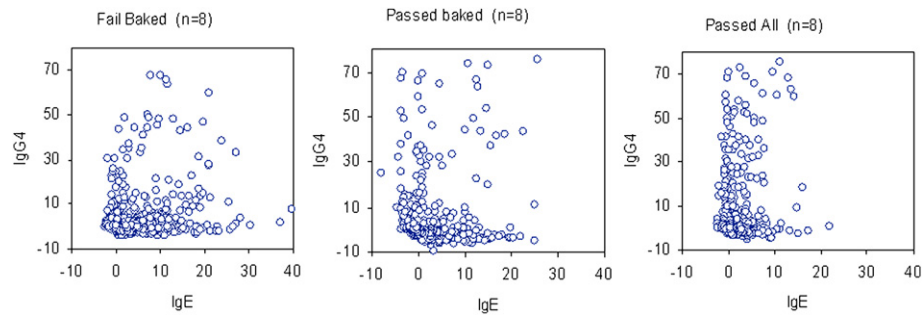


FIG E1. Scatter plot showing the correlation of IgE and IgG4 binding of 8 randomly selected subjects from each group. Each *spot* represents IgE binding (represented as a z score) to 1 milk peptide.

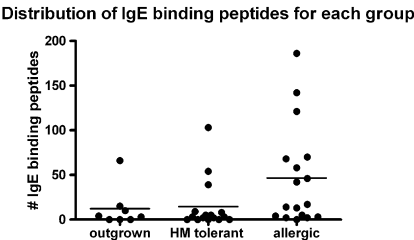


FIG E2. Distribution of IgE-binding peptides for each group.

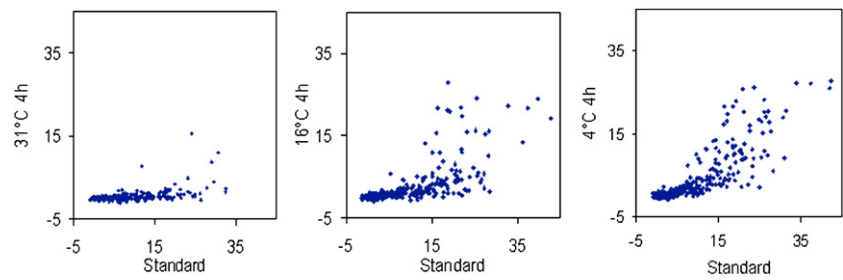


FIG E3. Effect of temperature. Scatter plot comparing IgE binding between the standard protocol (*x-axis*) and competition with 2 mg/mL caseins and 3 mg/mL β -lactoglobulin at different temperatures (*y-axis*) for 4 hours. Each *spot* represents IgE binding (represented as a z score) to 1 peptide of milk allergens.

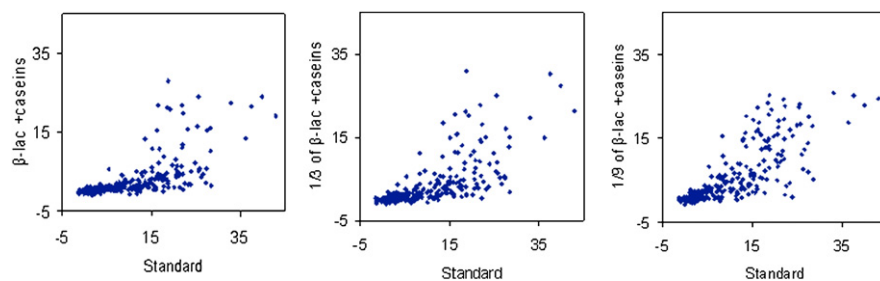


FIG E4. Effect of competitor concentration. Scatter plot comparing IgE binding of the serum pool between the standard protocol (*x-axis*) and competition with a one-third serial dilution of 2 mg/mL caseins and 3 mg/mL β-lactoglobulin (*y-axis*) at 16°C for 4 hours.

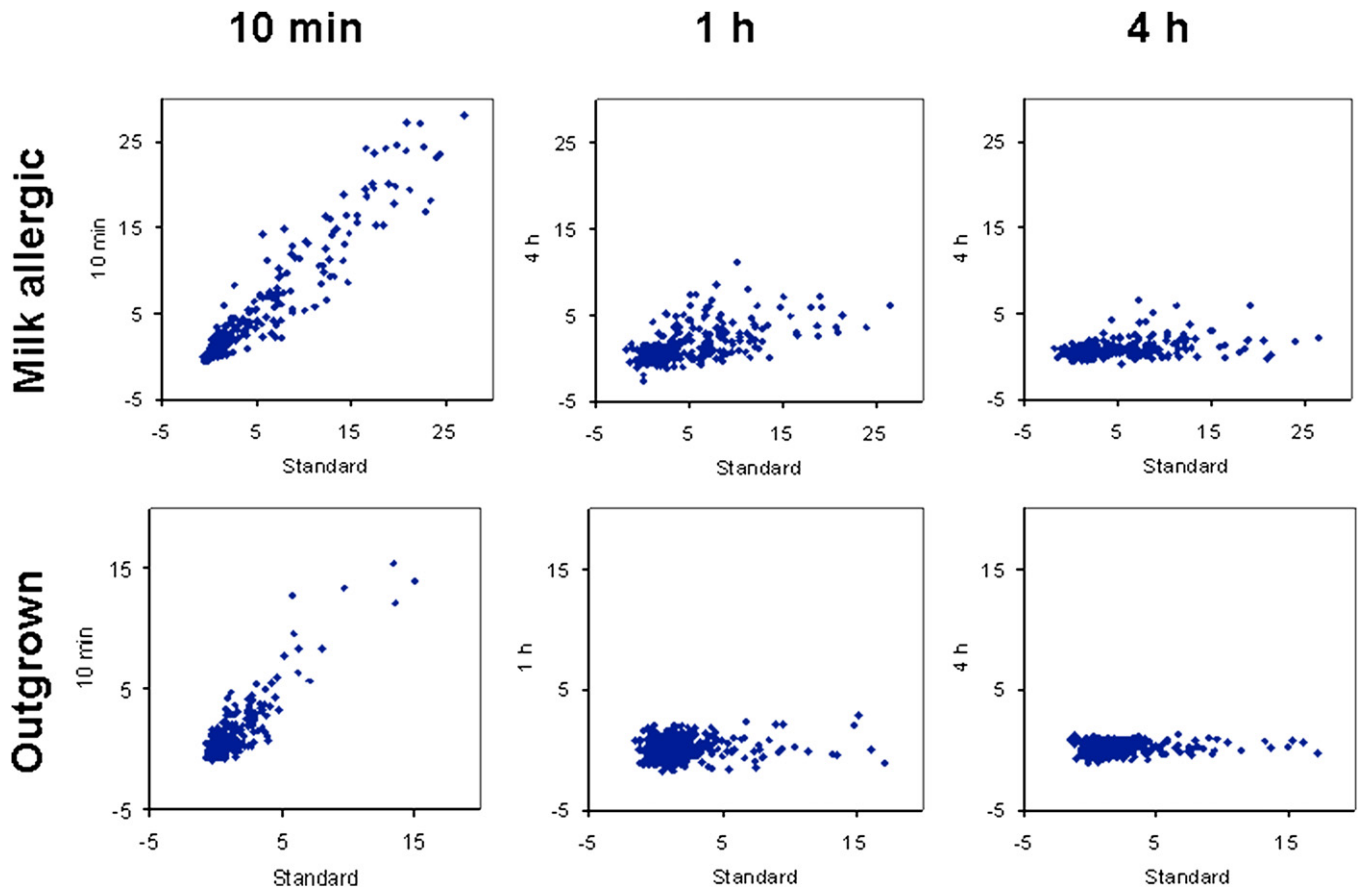


FIG E5. Effect of competition time. Scatter plot comparing IgE binding of a subject with milk allergy and a subject who had outgrown his or her allergy between the standard protocol (*x-axis*) and 1 mg/mL caseins and 1.5 mg/mL β -lactoglobulin (*y-axis*) at 16°C for different competition time.