

Original article

Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy

Background: Approximately two-thirds of egg-allergic infants become tolerant within the first 5 years of life.

Objective: We sought (1) to compare the recognition of sequential (linear) and conformational binding sites of ovomucoid, ovalbumin and ovotransferrin, by IgE antibodies of children with persistent and transient egg allergy, (2) to identify immunodominant IgE- and IgG-binding epitopes of ovomucoid, and (3) to compare epitope-specificity of IgE antibodies between patients with differing natural histories of egg allergy.

Methods: Using immunodot-blots or ImmunoCAPs, IgE-antibodies against conformational (native) and sequential (reduced and alkylated) egg proteins were determined at the time of clinical reactivity in patients who retained their allergy and in those who developed clinical tolerance. IgE- and IgG-binding epitopes were mapped for ovomucoid using overlapping decapeptides on SPOTs membranes. Recognition of the major IgE-binding epitopes were compared between patients with differing natural histories of egg allergy.

Results: The patients with long-lasting egg allergy had a higher concentrations of IgE antibodies against sequential and native ovomucoid and ovalbumin than the children who subsequently gained tolerance ($P < 0.01$). Four major IgE-binding epitopes were identified in ovomucoid at amino acid 1–10, 9–20, 47–56, and 113–124. IgE antibodies of all seven patients with persistent egg allergy recognized these epitopes whereas none of the 11 children who outgrew their egg allergy did so.

Conclusions: Patients with persistent egg allergy develop IgE antibodies against more sequential and conformational epitopes of ovomucoid and ovalbumin. The presence of serum IgE antibodies to specific sequential epitopes of ovomucoid may be used as a screening instrument for persistent egg allergy.

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Allergic reactions to foods affect up to 6% of children in the first few years of life (1), with cow's milk, egg, and peanut accounting for most of the documented allergic responses (2). Most milk-allergic children develop cow's milk allergy (CMA) in the first year of life and approximately 85% of them 'outgrowit' (becoming clinically tolerant) by 8.6 years of age (3). Hypersensitivity to hen's egg is more often recognized in the second year of life, and appears to be more persistent than milk allergy (4–6), with approximately half of the patients becoming tolerant in 3 years and up to 66% of children in 5 years (7). When egg-allergic children are placed on a diet devoid of all egg protein, about one-third develop clinical tolerance to egg within 2 years, even though IgE antibodies to egg (e.g. positive prick skin tests) persist for several years (5).

Egg allergy is present in about two-thirds of children with atopic dermatitis and food allergy (8, 9).

Allergic reactions are more frequently elicited by egg white proteins than egg yolk (10). Egg white contains 24 different glycoproteins. Ovomucoid (Gal d1), ovalbumin (Gal d2), ovotransferrin (Gal d3), and lysozyme (Gal d4), comprising 10%, 54%, 12%, and 3.5% of total egg white protein (11), have been identified as major allergens (12–14). However, ovomucoid has been shown to be the dominant allergen (15, 16), with ovotransferrin and lysozyme being less important (17). A recent paper reported that egg-allergic patients can be categorized into four distinct patterns, which may explain why various investigators have reported different allergens to be important in egg hypersensitivity (18). The importance of ovomucoid may be due to its unique characteristics such as relative stability against heat (20) and digestion with proteinases (19), and its strong allergenicity (15).

Abbreviations: AA, amino acid; OD, optical density.

compared with other egg white components. Ovomucoid is a 28 kDa glycoprotein comprised of 186 amino acids (AAs) arranged in three tandem domains containing nine intra-domain, but no inter-domain, disulfide bonds and five carbohydrate side-chains (20, 21). Utilizing pooled human sera from egg-allergic patients, five IgE and seven IgG-antibody binding sites have been identified dispersed along the three domains (22).

Studies addressing the mechanisms behind persistence of egg allergy, have shown that children with persistent egg allergy have significantly higher concentrations of IgE anti-ovomucoid antibodies than those who 'outgrow' their reactivity (15). Evaluation of IgE antibody binding to 'linearized' ovomucoid (i.e. reduced and alkylated) suggested that patients' ovomucoid-specific IgE antibodies differed in their ability to bind sequential and conformational epitopes (22). Sequential epitopes are determined by contiguous AAs, and conformational (discontinuous) epitopes are comprised of AA from different regions of the allergen brought in close proximity by folding of the polypeptide backbone. Urisu et al. showed that subjects with high IgE-binding activity to pepsin-treated ovomucoid are unlikely to outgrow egg white allergy (17), and in a more recent study, they attributed this activity to be likely due to a small 4.5 kDa fragment of a pepsin-digested ovomucoid (23).

Our previous studies on cow's milk proteins suggested that differences in epitope-specificity of IgE antibodies against cow's milk proteins play a role in persistence of CMA (24, 25). We showed that six epitopes (three on α_{s1} -casein, one on α_{s2} -casein, and two on κ -casein) were not recognized by IgE antibodies from any of the patients who 'outgrew' their CMA, but showed binding by the majority of the patients with persistent allergy. This might reflect differences in antigen absorption and processing, and measurement of these specific IgE antibodies could be used as a marker of persistent CMA.

The present study sought to investigate the qualitative differences in the recognition of sequential and conformational IgE-binding epitopes of major egg proteins. We also sought to determine whether these differences are associated with the natural history of egg allergy.

Methods

Determination of specific IgE binding to native and linearized egg proteins

Individual egg proteins (ovomucoid, ovalbumin and ovotransferrin; Sigma, St Louis, MO, USA) were dissolved in PBS. Half of each protein solution was 'linearized' (reduced and alkylated) by adding dithiothreitol at a final concentration of 0.11 M and boiling the mixture for 2 min. The solutions were then saturated with nitrogen and capped, and the reaction was allowed to proceed for 3 h at 37°C. Iodoacetamide was added to a final concentration of 0.25 M and the pH was adjusted to 8.5. After 30 min at room temperature, β -mercaptoethanol was added to each solution to a final concen-

tration of 1 M. Finally, the samples were dialysed against PBS overnight at 4°C and then stored -20°C until further use. A change of conformation after reduction and alkylation was confirmed by tris-glycine-bis-acrylamide gel electrophoresis using 10–20% gradient gel (NuPage; Novex, San Diego, CA, USA), without reducing agents (native gel). After 1.5 h at 125 V constant voltage, staining was performed with Coomassie blue (Fig. 1).

For determination of specific IgE antibodies binding to ovomucoid, both linearized and native ovomucoid were titrated to optimal concentrations and conjugated to the solid phase of ImmunoCAP™ by Phadia AB, Uppsala, Sweden. Serum IgE antibody-binding was measured by CAP System FEIA (Phadia) and results are expressed as kU_A/l.

For measurement of specific IgE antibodies to ovalbumin and ovotransferrin, the immunodot-blot method was used. Therefore, 10 μ g per well of native and linearized ovalbumin and ovotransferrin in 100 μ l of PBS was applied to nitrocellulose membrane with a 96-well minifold dot-blotter apparatus (Schleicher & Schuell Inc., Keene, NH, USA). After blocking with 0.5% porcine gelatin in PBS at room temperature and washing with PBS-0.05% Tween 20, the dots were incubated for 2 h at room temperature, in duplicate, with patients' sera diluted 1 : 10 in 0.5% porcine gelatin/PBS. The dilution factor was based on preliminary experiments resulting in a clear labeling of the dot-blot by low background levels. After washing, dot-blot was incubated with goat IgG anti-human IgE conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) in a blocking buffer. After the final wash, dots were rinsed with 50 mmol of TRIS,

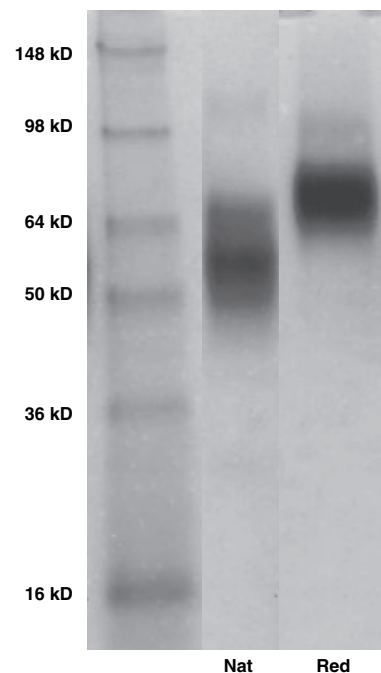


Figure 1. Modification of ovomucoid. Ovomucoid was linearized using dithiothreitol, iodoacetamide, and β -mercaptoethanol. Electrophoresis was performed using 10–20% gradient tris-glycine bis-acrylamide gel without reducing agents (native gel). Staining was performed with Coomassie blue. The first lane corresponds to the molecular weight standard, and other lanes correspond to native (N) and reduced and alkylated (RED) ovomucoid (OM).

pH 9.6, for 2 min and developed with one component BCIP/NBT phosphatase substrate (KPL). The reaction was stopped with distilled water. The optical density (OD) of each individual spot was measured.

For comparing the recognition of conformational and sequential egg proteins by IgE antibodies from patients with different natural histories of egg allergy, two different subpopulations were identified. In all the patients, egg allergy was initially diagnosed by double-blind, placebo-controlled food challenge or by a history of a severe reaction to isolated ingestion of egg. The first group included 21 patients with persistent egg allergy. In the follow-up, the patients had remained clinically reactive to egg beyond the median age of 11 years (range 7–18 years). Sera used for testing was drawn at 2.5–16 years (median 5 years) of age. Egg-specific serum IgE antibody levels at this time were median 61.3 kU_A/l (range 2.64–1060 kU_A/l), as measured by ImmunoCAP. One of the patients reacted with anaphylaxis; nine developed mucocutaneous symptoms (pruritic rash, urticaria, mouth/throat itching), nine developed vomiting and mucocutaneous symptoms, and two developed laryngeal edema/respiratory distress. The second group included 16 patients who outgrew egg allergy at the median age of 5 years (range 4–18). At the time of blood drawn for testing, they were still clinically reactive to egg and their median age was 3 years (range, 4 months to 15 years) with median egg-specific IgE level 4.6 kU_A/l (range, 0.47–234). Following exposure to egg, one developed anaphylaxis, 12 of the patients developed mucocutaneous symptoms, two developed vomiting and mucocutaneous symptoms, and one developed abdominal pain.

Informed consent was obtained from all patients/guardians included in this study, and the study was approved by the Institutional Review Board of the Mount Sinai School of Medicine.

Mapping of IgE and IgG epitopes by SPOTs membrane using individual patient sera

SPOTsTM membrane (Genosys Biotechnologies, Woodlands, TX, USA), a derivatized cellulose membrane, was used to generate 89 decapeptides, overlapping by eight AAs and covering the entire sequence of ovomucoid. Individual peptides were synthesized on the membrane by the 9-fluorenyl-methoxycarbonyl method according to the manufacturer's instruction. Cycles of coupling, blocking and deprotection were repeated until the peptides of the desired length were synthesized. Peptide synthesis reactions were monitored by bromophenol blue color reactions during certain steps of the synthesis.

The membranes were blocked overnight with 0.5% porcine gelatin in PBS containing 0.01% Tween 20, and 1% human serum (from a donor with no detectable IgE to egg proteins). Individual patients' sera from 23 patients with detectable levels of egg-specific IgE antibodies (median 80.4 kU_A/l, range 1.66 to >100 kU_A/l) were diluted 1 : 12 for IgE binding and 1 : 100 for IgG binding, and membranes were incubated with diluted sera at room temperature for 2 h. Control sera were obtained from five atopic, nonegg-allergic individuals with atopic dermatitis for both IgE and IgG binding. They had negative prick skin tests to egg and/or egg-specific IgE antibodies <0.35 kU_A/l, and clinical tolerance to egg. An additional seven healthy, nonatopic individuals were included as controls for IgG binding. After washing in PBS-0.05% Tween 20, the membrane was incubated with biotin-conjugated goat anti-human IgE followed by avidin–horseradish peroxidase (HRP, KPL). For detecting patients' IgG antibodies, rabbit anti-human IgG-HRP conjugate (Dako Corp, Santa Barbara, CA, USA) was used. The membranes were developed with the ECL chemiluminescent HRP detection kit (Amersham, Arlington

Heights, IL, USA), and exposed to an X-ray film. A new SPOTs membrane was generated for probing every 10 patients' serum samples, and normal human serum was used for testing between patient each patient sample determination to ensure that all membranes had been completely stripped of IgE or IgG antibodies from the previous subject.

After developing the X-ray film, OD of each individual peptide spot was measured by reflection densitometry. The OD of each peptide spot was recorded as the difference between the OD of the peptide spot and the OD of background of the film. Each decapeptide was assigned a 'cumulative score,' which represents the sum of ODs of all patients in each group. The cumulative scores and individual scores from each patient were used to identify particular regions in the protein that were recognized by patients as a group and by individual patients. Major IgE and IgG binding regions were defined as regions bound by IgE and IgG antibodies from at least 50% of the patients. The cut-off level for significant binding was an OD of 0.1 for individual sera.

Comparison of recognition of ovomucoid by persistent and tolerant patients

Four decapeptides representing the major IgE-binding epitopes were synthesized on SPOTs membrane as described above. As SPOTs method is work-intensive, membranes were labeled with individual patient sera from seven patients with persistent egg allergy and 11 patients with transient egg allergy, randomly selected among the two subpopulations described earlier, using alternatively iodine 125-conjugated goat anti-human IgE antibody. These patients represented a subpopulation of the patients used for immunodot-blot and ImmunoCAP measurements as described above. Sera from three nonegg-allergic individual were used as negative controls.

Statistics

The Mann–Whitney *U*-test was used to compare results between persistent or tolerant egg-allergic patients, and the Wilcoxon signed rank test was used to compare measurements in the same patients at different time points. Analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Specific IgE antibody binding to native and linearized egg proteins in persistent and transient allergy

Concentrations of specific IgE antibodies against native (conformational) and linearized (reduced and alkylated) ovomucoid were determined by ImmunoCAP using sera from 21 children who in the follow-up period retained their allergy and 16 children who developed clinical tolerance. Children with persistent allergy had significantly higher levels of IgE against linearized ($P < 0.01$) and native ovomucoid ($P < 0.01$) than those with transient allergy, as shown in Figs 2A and B. In addition, in a subpopulation of these children, specific IgE binding to native (intact conformation) and linearized ovalbumin and ovotransferrin was determined in nine children with persistent egg allergy and 10 patients who subsequently became clinically

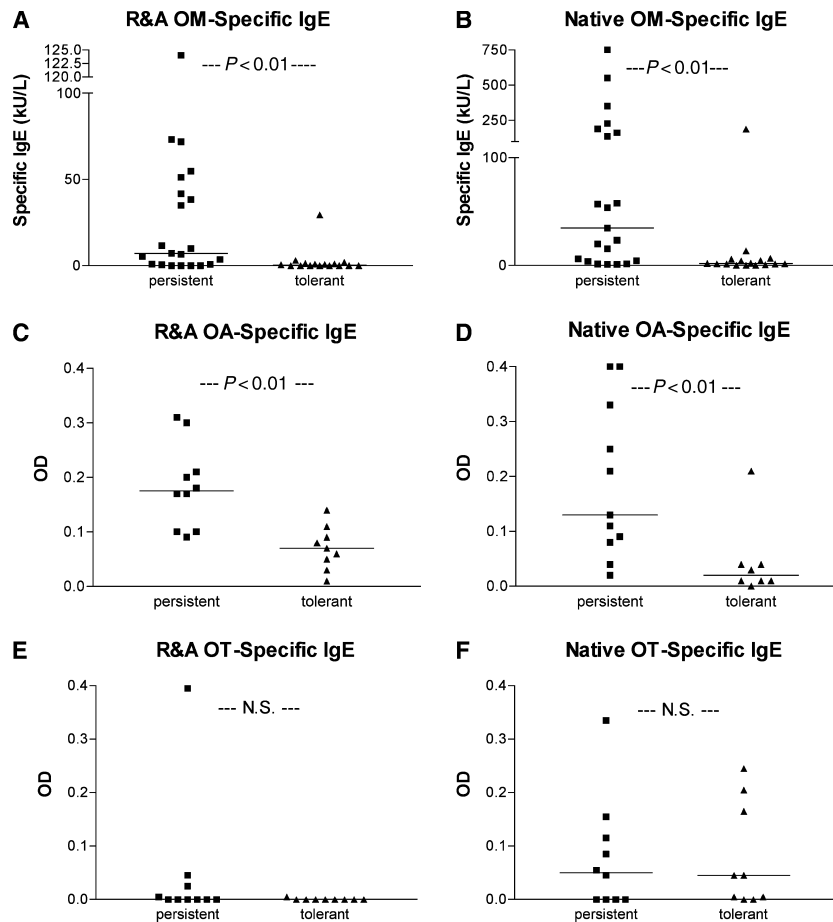


Figure 2. Comparison of specific IgE antibody binding to linearized (reduced and alkylated, R&A) and native ovomucoid (OM) (A and B, respectively), linearized and native ovalbumin (OA) (C and D), and linearized and native ovotransferrin (OT) (E and F) in children with persistent egg allergy and in those who gained clinical tolerance. For ovomucoid, linearized and native proteins were applied to a solid matrix and specific IgE-binding was tested using the CAP System FEIA. For ovalbumin and ovotransferrin, the proteins were applied to a nitrocellulose membrane and tested for specific IgE binding using immunodot-blot. In all children the sera were obtained at the time of clinical reactivity. The horizontal line represents the median. OD, optical density.

tolerant, using immuno-dot blot analyses. Children with persistent egg allergy had a higher level of specific IgE antibodies to linearized ($P < 0.001$) and native ovalbumin ($P < 0.01$) (Figs 2C and D). However, the level of IgE antibodies against linearized ($P = 0.24$) and native ovotransferrin ($P = 0.84$) was comparable in patients with persistent and transient egg allergy (Figs 2E and F).

Mapping of linear IgE and IgG binding epitopes of ovomucoid

In order to compare epitope recognition between persistent and transient egg-allergic patients, we first determined the sequential IgE and IgG-binding epitopes of ovomucoid. Sera from 23 patients with elevated egg white-specific IgE antibodies were randomly chosen for individual evaluation with overlapping decapeptides of ovomucoid synthesized on a cellulose-derivatized membrane. The sum of OD of individual patients' IgE antibodies binding to each decapeptide is shown in

Fig. 3A. The regions recognized by at least 50% of the patients were defined as major epitopes. Three major IgE-binding epitopes are located in the first domain, and the fourth major epitope was in the second ovomucoid domain. In addition, 10 areas were recognized by six to eight of the 23 patients (Fig. 3A). The median number of major epitopes recognized per patient was 1 (range 0–4). The atopic, nonegg-allergic controls showed no binding to the peptides.

Individual sera from 17 of the patients used for studying IgE binding were randomly selected and used for mapping IgG-binding regions. Twelve controls (five atopic individuals with atopic dermatitis and seven nonatopic ones) with egg-specific IgE < 0.35 kU/l and clinical tolerance to egg were used as controls. Figure 3B shows the cumulative OD of IgG binding of egg-allergic patients to each of the peptides, with the number of patients and controls binding to each region shown in parentheses.

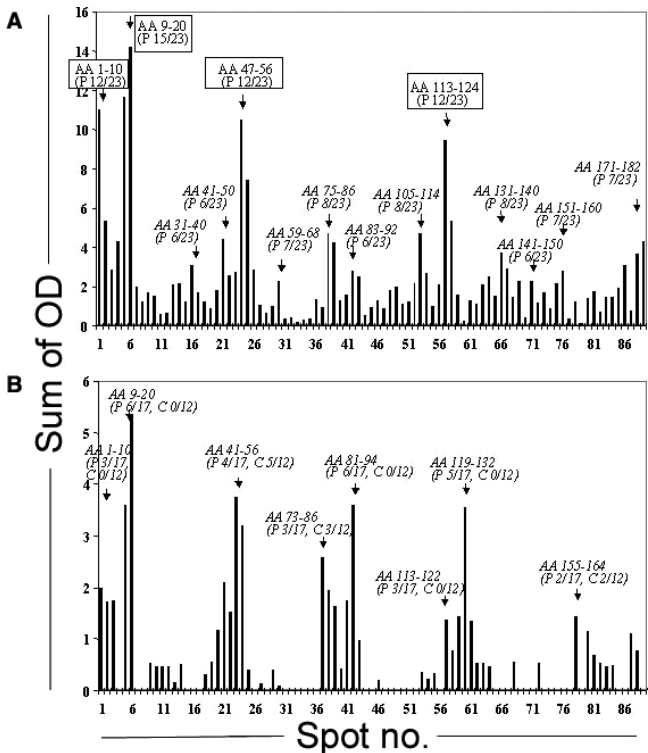


Figure 3. Cumulative optical density (OD) scores for specific IgE to each of the synthetic decapeptides of ovomucoid. According to the known amino acid (AA) sequence, 89 overlapping decapeptides were synthesized on a cellulose-derivatized membrane. Individual sera from 23 egg-allergic children were used to identify IgE-binding epitopes (A), and sera from 17 patients were used to identify IgG-binding epitopes (B). The epitopes and their corresponding AA sequence are presented above the bars. The major binding sites are shown in the boxes and the minor ones in italics. The number of patients (P) and controls (C) recognizing each epitope are shown in the brackets.

Comparison of recognition of sequential IgE epitopes of ovomucoid in persistent and transient allergy

Given the lower level of linearized ovomucoid-specific IgE antibodies as measured by ImmunoCAP in patients who outgrew their allergy, we sought to identify the specific differences in the recognition of sequential IgE-binding epitopes when compared with the patients with persistent egg allergy. For this purpose, the central decapeptides from each of the major IgE-binding epitopes identified was selected for further synthesis on a SPOTs membrane as follows: AA 1–10, 11–20, 47–56, and 113–122. These epitopes were then labeled with individual sera from 11 patients (egg-specific IgE ranging from 0.69 to 234 kU_A/l) who subsequently had lost clinical reactivity to egg in the follow-up period, and the results were compared with the IgE binding of seven patients (egg-specific IgE ranging from 2.64 to 1060 kU_A/l) who retained their clinical reactivity. None of the patients with transient egg allergy had IgE antibodies against

these epitopes, whereas all of the seven patients with persistent allergy possessed such antibodies (Fig. 4). Table 1 summarizes these ‘informative’ epitopes, which were recognized by the majority of patients with persistent egg allergy but by none of the transient egg-allergic patients. Search for IgE antibodies against these informative epitopes may be useful in identifying all egg-allergic patients who will retain their allergy.

Discussion

In the present study, we have demonstrated that qualitative differences in the recognition of sequential and conformational IgE-binding epitopes of ovomucoid and ovalbumin are found in the patients with differing natural histories of egg allergy. In general, patients with persistent egg allergy develop more IgE antibodies against sequential and conformational structures of ovomucoid and ovalbumin than the patients who will subsequently gain clinical tolerance. We also demonstrated that epitope-specificity of IgE antibodies is associated with the development of clinical tolerance to ingested egg protein, and suggest that the presence of serum IgE antibodies to certain sequential ‘informative’ epitopes of ovomucoid may be used as a screening test to identify patients with persistent egg allergy.

IgE antibodies produced by B cells activated following surface-IgM binding to exposed oligopeptides on the native protein may be directed at sequential epitopes

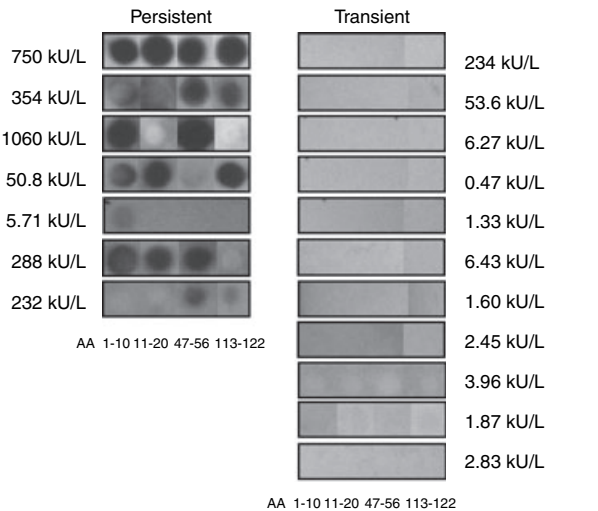


Figure 4. Binding to the four major IgE-binding decapeptides of ovomucoid by serum IgE-antibodies of seven patients with persistent egg allergy (left panel) and by 11 patients who achieved clinical tolerance (right panel) using SPOTs membranes. In all the patients, serum was obtained at the time of clinical reactivity. Numbers below the panels indicate the amino acid corresponding to the peptide. Egg-specific IgE antibody levels of each patient are shown.

Table 1. Residues and corresponding amino acid sequences of the 'informative epitopes' in ovomucoid, which were recognized by serum IgE-binding epitopes of all seven children with persistent egg allergy, but by none of the 11 children with transient egg allergy

Amino acid	Sequence	No. patients binding	
		Persistent	Tolerant
1–10	AEVDCSRFPN	6/7	0/11
11–20	ATDKEGKDV	4/7	0/11
47–56	SIEFGTNISK	5/7	0/11
113–122	VEQGASVDKR	6/7	0/11

determined by contiguous AAs or conformational epitopes, comprised of AA residues from different regions of the allergen brought into proximity by folding. Both sequential and conformational B cell epitopes have been identified to inhaled aeroallergens (26, 27), although the latter appear to predominate. As food allergens are subjected to extensive chemical and proteolytic digestion prior to absorption and uptake by cells of the gut-associated lymphoid tissue, it has been inferred that food allergenic epitopes are predominantly sequential in nature. In previous studies, evaluation of reduced and alkylated, i.e. 'linearized' ovomucoid suggested that not all patients had anti-ovomucoid antibodies that recognized sequential epitopes (22). This prompted the question of whether epitope-specificity of anti-egg-IgE antibodies could differ in patients with differing natural histories of egg allergy.

In the present study, we investigated the presence of specific IgE antibodies against sequential and conformational epitopes of egg proteins. We found that at the time when patients were first found to have clinical hypersensitivity, those with long-lasting egg allergy had relatively larger proportions of IgE antibodies directed at 'linearized' ovomucoid and ovalbumin than to conformational protein compared with the children who subsequently gained clinical tolerance. This detected difference in specificity of IgE responses between these patient groups may reflect differences in digestion, absorption, and/or processing of egg proteins. Infants have been shown to possess increased levels of food proteins in the circulation following meals (28). This is felt to be secondary to maturational delay in the development of digestive processes, e.g. stomach acidity, proteolytic enzyme activity, mucin composition, etc., and consequent increased uptake (2). The more permeable infant gut would allow significant quantities of macromolecular antigen (and more likely conformationally intact food protein) to gain access to local B cells that upon activation generate egg-specific IgE antibodies in genetically predisposed hosts. With maturation of the gastrointestinal tract, less conformationally intact protein would be accessible (28) to activate gut-associated lymphoid tissue and IgE-bearing mast cells, resulting in loss of clinical reactivity and eventual loss of allergen-specific

IgE antibody synthesis. Complete dietary exclusion of egg protein would further promote loss of clinical reactivity, whereas continued exposure to minute amounts of egg protein fragments (containing sequential epitopes) would continue to penetrate the gastrointestinal barrier (3, 29), and result in the continued synthesis of IgE antibodies to sequential egg epitopes in susceptible children and consequently persistent egg reactivity. This is consistent with the observation that the likelihood of losing clinical reactivity is associated with the age of the patients at the time of diagnosis, the degree of avoidance of the responsible allergen, and the allergen in question, e.g. peanut compared with egg. The younger the patient at the time of food sensitivity is diagnosed and/or the more stringent the allergen avoidance, the more likely it appeared that the patient 'outgrew' his/her food allergy (4, 5, 30).

We also utilized overlapping, linear decapeptides to identify IgE-binding epitopes of ovomucoid using individual sera from 23 patients with high levels of egg-specific IgE antibodies. Using pooled sera from seven egg-allergic patients, we previously identified five IgE-binding epitopes of ovomucoid (22), which also were identified in this study. Holen et al. (31) more recently mapped IgE-binding regions by using individual sera from seven patients, and reported four epitopes recognized by more than half of the patients and three that were each recognized by fewer patients. Interestingly, we found new allergenic areas at AA 41–50, 59–68, 131–140 and 151–160, the first two of which were identified as T-cell epitopes, but not IgE-binding epitopes, by Holen et al. (31). In the present study, mapping of antigenic (IgG binding) areas showed binding in the same areas as IgE binding in the first and second domain, whereas the antigenic areas (AA 119–132 and 155–164) differed from allergenic regions (131–140, 151–160, and 171–182) in the third domain. None of the antigenic areas were immunodominant and also were recognized by occasional healthy controls.

A comparison of ovomucoid epitope-specificity of IgE antibodies showed striking differences in the patients with transient and persistent egg allergy. Four IgE-binding epitopes were not recognized by any of the patients with transient egg allergy whereas they were recognized by all the children with persistent hypersensitivity to egg protein. These epitopes were therefore designated as 'informative' epitopes and detectable levels of specific IgE antibodies against a combination of these epitopes could serve as a screening test for persistent egg allergy. This result further supports the concept of differences in the antigen absorption and processing between patients with different outcomes of egg allergy.

In conclusion, we found that the development of IgE antibodies against specific sequential epitopes of ovomucoid is associated with persistence of egg allergy. We identified four sequential IgE-binding sites on ovomucoid that differentiated between patients with persistent egg

allergy and those with transient egg allergy. The presence of IgE antibodies against a combination of the 'informative' epitopes may be useful as a marker of persistent egg allergy. As IgE antibodies to these 'informative' epitopes appear to develop early in life, this approach provides the advantage of allowing screening at the time of initial diagnosis in infants. Studies are ongoing linking 'informative' epitopes (peptides) to a commercial matrix in order to conduct prospective studies in larger patient populations.

Acknowledgments

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