Role of conformational and linear epitopes in the achievement of tolerance in cow’s milk allergy

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Summary

Background  Cow’s milk (CM) is one of the leading causes of food allergy in children. However, approximately 85% of milk-allergic children become clinically tolerant to CM within the first 3 years of life. The mechanisms involved in the achievement of tolerance remain unknown.

Objective  To study whether IgE antibodies from children with persistent cow’s milk allergy (CMA) differ from children who become clinically tolerant in their ability to recognize linear and conformational epitopes of \( \alpha_s1- \) and \( \beta \)-casein.

Methods  Thirty-six milk-allergic children were included in the study: 11 of the children became clinically tolerant, and 25 had persistent CMA. Blood was obtained from all patients during the time they showed clinical reactions to milk challenge. Six non-milk-allergic children served as controls. Specific IgE antibodies against linear (denatured) as well as conformational (native) milk proteins were determined by probing dot-blots with patients’ sera. In addition, selected decapeptides from \( \alpha_s1- \) and \( \beta \)-casein, previously found to be suggestive of persistent CMA, were synthesized on a cellulose-derivatized membrane and probed with individual sera from 10 patients who outgrew CMA and from 10 patients with persistent CMA.

Results  Analysis of immunodot-blots showed that, in comparison to tolerant patients, milk-allergic children with persistent symptoms had a significantly higher ratio of specific IgE antibodies to linearized than to native \( \alpha \)- and \( \beta \)-casein (\( P < 0.005 \) and \( P < 0.02 \), respectively). Comparing the selected decapeptides, six of the 10 patients with persistent allergy recognized the peptide corresponding to amino acids 69–78 from \( \alpha_s1- \)-casein while none of the patients who outgrew CMA had IgE binding to this epitope. Conclusion  Patients with persistent milk allergy possess higher detectable levels of IgE antibodies to linear epitopes from \( \alpha_s1- \) and \( \beta \)-casein than children who have achieved tolerance. Specific IgE binding to particular linear epitopes in \( \alpha_s1- \)-casein may be a predictive factor for persistence of CMA.

Keywords: cow’s milk allergy, tolerance, epitope, conformational, linear, \( \alpha_s1- \)-casein, \( \beta \)-casein.

outgrow their clinical allergy. Further remission of CMA may occur in up to one-third of older children following 1–2 years of strict avoidance of cow’s milk [4]. The mechanisms responsible for the development of tolerance remain poorly understood.

Several studies have focused on finding markers to predict whether children are going to remain allergic or to outgrow CMA [5–8]. Some of these studies have shown that children with long-lasting CMA present higher levels of total [5] and specific [6] IgE to cow’s milk (CM) than those who became tolerant. Furthermore, lack of reactivity to particular protein fractions of CM may be directly related to the achievement of tolerance. James and Sampson observed that casein- and β-lactoglobulin-specific IgE concentrations and the IgE : IgG ratio were significantly lower in children who ‘outgrew’ their milk reactivity, suggesting that monitoring these parameters might be useful in predicting the natural history of CMA [7]. Sicherer and Sampson reported that children with persistent CMA showed significantly higher levels of specific IgE to whole milk and casein than those children younger than 3 years of age who were likely to outgrow their allergy [8].

Interestingly, some children who are allergic to milk and egg are able to tolerate small amounts of these foods in a cooked, but not in a raw, form [9,10]. During food processing, the native structure and many conformational epitopes of these proteins are modified or disrupted by heat and/or chemical treatments, eliminating IgE binding to conformational epitopes and exposing linear epitopes. Cooke and Sampson observed a different pattern of specific IgE reactivity to linear and conformational epitopes of ovomucoid between children with persistent egg allergy and those likely to outgrow it [11]. They suggested that the development of IgE antibodies to linear epitopes may be related to the persistence of allergy to egg [11]. We recently identified IgE and IgG binding epitopes of αs1-casein in CM-allergic patients and showed a different pattern of IgE binding in older patients with long-lasting CMA and in the younger children likely to outgrow their allergy [12].

In this study we compare binding of specific IgE to the main protein fractions in cow’s milk, both in a native and a linearized form, in children who clinically outgrew milk allergy (using sera from the time of initial evaluation when patients were clinically reactive) and children who remained reactive to cow’s milk. We also sought to identify linear epitopes that would allow us to differentiate between these two groups of children, which could help in predicting the natural history of CMA in individual patients.

Methods

Subjects

Thirty-six children were included in this study: 11 patients who had outgrown milk allergy and 25 with persistent CMA. Children with milk allergy for at least 7 years were considered to have persistent CMA. Of the 25 patients with persistent allergy, 11 had a positive double-blind, placebo-controlled food challenge (DBPCFC) with immediate type symptoms between the ages of 7 and 15 years (median age 8 years). Three others had an accidental exposure to milk at the ages of 7–12 years and milk-specific IgE concentrations > 100 kU/L, as determined with the CAP System FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). These three children developed urticaria immediately after milk ingestion and one patient also developed respiratory symptoms. Of the remaining 11 patients, four initially had a positive DBPCFC with immediate type symptoms at the ages of 3–6 years (median age 5 years) and had milk-specific IgE between 33.7 and > 100 kU/L (median 74.3 kU/L) at ages 7–12 years, indicating greater than 95% likelihood for clinical reactivity to milk [13]. The remaining seven patients had accidental ingestion at 1–4 years of age (median age 2 years), five of them with anaphylaxis, two with severe immediate gastrointestinal symptoms or urticaria. Between the ages of 7 and 9 years, the seven patients had milk-specific IgE levels between 25 and > 100 kU/L, again indicating greater than 95% likelihood of clinical reactivity [13].

Of the 11 patients who outgrew their milk allergy, three had sequential DBPCFC, first with positive immediate reactions and then 2 to 6 years later with negative challenges. The other eight had convincing clinical history with objective immediate type reactions (vomiting, urticaria, respiratory symptoms or angioedema) after milk ingestion. One to 6 years (median 5 years) after the initial diagnosis of their allergy, these patients no longer showed clinical reactions during DBPCFC.

Six atopic children sensitized to inhalant and/or food allergens other than CM served as controls. In all children, blood was collected at the time of clinical reactivity. Serum samples were stored at −70 °C.

Preparation of milk proteins

Individual milk proteins (α-lactalbumin, β-lactoglobulin, α-casein and β-casein, Sigma, St Louis, MO, USA) were dissolved in phosphate-buffered saline (PBS). Half of each protein solution was ‘linearized’ by adding dithiothreitol (DTT) 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 (23°C), 2β-mercaptoethanol was added to each solution to a final concentration of 1 M. Finally, the samples were dialysed...
against PBS overnight at 4 °C and then stored at −20 °C until further use.

To test the successful modification of the proteins, tris-glycine-bis-acrylamide gel electrophoresis was performed using 10–20% gradient gel (NuPage, Novex, San Diego, CA, USA), without reducing agents (native gel). After 1.5 h at 125 V constant current, staining was performed with Coomassie blue.

**Immunodot-blots**

Ten micrograms per well of each native and linear protein in 100 µL 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/PBS at room temperature and washing with PBS-Tween 0.05%, the dots were incubated for 2 hours 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 labelling of the dotblots by low background levels. After extensive washing, dot blots were 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, pH 9.6, for 2 min and developed with one component BCIP/NBT phosphatase substrate (Kirkegaard and Perry Laboratories). The reaction was stopped with distilled water.

Immunodot-blots were analysed by measuring the optical density (OD) for each dot, with a reflection densitometer (Macbeth, Newburgh, NY, USA). The ratio of IgE binding to linear vs. conformational milk proteins was obtained for each patient to avoid false conclusions due to the differences in specific IgE levels between individuals. Patients with negative OD for native proteins were excluded for the particular protein, since in those cases the ratio could not be calculated.

**SPOTs membrane for α-casein and β-casein epitopes**

In previous studies, IgE binding epitopes for αs1-casein and β-casein were identified after synthesis of overlapping decapeptides using the SPOTs kit (Genosys Biotechnologies, Woodlands, TX, USA). Comparing older patients with persistent allergy and younger ones likely to outgrow their CMA, two IgE binding regions in αs1-casein [12] and four in β-casein were usually recognized by the older patients (unpublished observations).

The central decapeptide from each epitope was selected for further synthesis on a SPOTs membrane as previously described [11]. For αs1-casein: spot A-I corresponds to amino acids 69–78 and spot A-II corresponds to amino acids 177–186. For β-casein: spot B-I corresponds to amino acids 151–160, spot B-II corresponds to amino acids 167–176, spot B-III corresponds to amino acids 175–184 and spot B-IV corresponds to amino acids 193–202. These peptides were then screened with individual sera from 10 patients with persistent CMA and with sera from 10 patients who outgrew CMA, using sera of the time of their clinical reactivity. After blocking with 1.5% human serum albumin (Sigma) overnight at 4 °C, SPOTs membrane was incubated for 10 h at 4 °C with individual patient sera. A serum dilution of 1 : 5 was used for each individual who outgrew his/her cow’s milk allergy. Even with the low levels of specific IgE in this patient group, specific antibody binding to decapeptides was demonstrated in preliminary experiments. To adjust for the higher IgE levels in the patients with persistent CMA, usually about 10-fold that seen in the tolerant group, sera were diluted 1 : 50. After washing three times in PBS-T for 7 min, the membrane was incubated with biotin-conjugated goat anti-human IgE antibody followed by avidin-horseradish peroxidase (Kirkegaard and Perry Laboratories). The membrane was developed with the chemiluminescent detection system (Amersham, Arlington Heights, IL, USA). Non-specific binding to the SPOTs membrane was ruled out by probing the membrane with sera from non-milk-allergic donors.

**Statistics**

The Mann–Whitney U-test was used to compare the ratios of IgE to ‘linearized’ vs. ‘native’ proteins between milk-tolerant and persistent milk-allergic patients. Analyses were performed with GraphPad Prizm (GraphPad Software, San Diego, CA, USA).

**Results**

**Modification of the native structure of cow’s milk proteins**

In order to obtain linear epitopes to test for IgE-binding, native α-casein, β-casein, α-lactalbumin and β-lactoglobulin were denatured. DTT was used to linearize α-lactalbumin, β-lactoglobulin and the αs2-casein by breaking their disulphide bonds. Iodoacetamide was further added to prevent linearized milk proteins from folding back to their native secondary and tertiary structure. β-Casein and the αs1-casein lack disulphide bonds and their spatial conformation is stabilized by hydrophobic interactions. DTT at high concentration is able to disrupt those interactions, leading to a more linear form of these proteins.

Structural changes following reduction and alkylation are suggested by the different behaviour of native and linearized proteins during electrophoresis on native gel (Fig. 1). Each native milk protein appears primarily as a
sharp band, while denatured proteins appear as wider, more diffuse bands. Interestingly, native proteins seem to migrate faster on the gel.

**Immunodot-blot**

In order to compare specific IgE binding to conformational and linear milk proteins between children with persistent milk allergy and children who became clinically tolerant, immunodot-blot analyses were performed. The ratio of linear and conformational protein was used for statistical analyses, as patients who outgrew CMA had significantly (P < 0.001) lower levels of specific IgE to CM (median 2.84 kU/L) than patients with persistent CMA (median 100 kU/L). Children with persistent CMA showed significantly higher ratios of specific IgE antibodies to linear vs. conformational epitopes to α- and β-casein than the patients who outgrew their allergy (P < 0.005 and P < 0.02, respectively, Figs 2a and b). Specific serum IgE antibodies to native α-lactalbumin were found in only two of the tolerant patients and eight of 25 patients with persistent milk allergy. In similar studies with β-lactoglobulin, none of the children developing tolerance and nine of 25 children with persistent CMA possessed IgE antibodies to linearized protein. The non-milk-allergic controls showed no binding to any of the milk proteins tested.

**Discussion**

Although it has been estimated that more than 80% of children allergic to CM will become clinically tolerant by the age of 3 years, the underlying mechanism remains unknown [1]. In this study we were able to demonstrate that high levels of detectable IgE to linearized α-casein are related to persistence of CMA. Moreover, intensive binding of IgE to specific epitopes of αs1-casein seems to be a common feature of children with persistent CMA, but not in those who clinically outgrow their allergy.

Since persistent cow’s milk allergy is rare, one critical element of the present study is to prove the persistency of milk allergy in the present study population. The 14 children with either positive DBPCFC (n = 11) or accidental allergic reactions and high IgE levels (n = 3) between the ages of 7 and 15 years have ‘definite persistent milk allergy’. In the remaining 11 patients, the diagnosis of persistent cow’s milk allergy was based on high specific IgE levels between 7 and 12 years of age. It was shown previously that children with cow’s milk-specific IgE levels in this range have greater than a
95% chance of clinical reactivity [13]. However, utilizing subgroup analysis of the 14 patients with ‘definite persistent milk allergy’ would result in the same significant differences between children with persistent and transient cow’s milk allergy by immunodot-blot as reported for the whole group. With respect to the SPOTs membrane, 8 of the 10 persistent cow’s milk-allergic patients fall into the group of ‘definite persistent milk allergy’ and only in patients K and O was the diagnosis of persistent milk allergy based on high IgE levels. There would be no differences in the results if only the 14 patients with ‘definite persistent milk allergy’ were included in the analysis.

Caseins account for about 80% of the total protein content in milk, whereas whey proteins represent 20%, the major fractions being α-lactalbumin and β-lactoglobulin. There are four proteins in the casein fraction: αs1-, αs2-, β- and κ-caseins, comprising 32%, 10%, 28% and 10% of the total protein content, respectively [14]. αs1-casein and αs2-casein are coded by different genes that are present on the same chromosome, and share only 30% amino acid sequence homology (http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi). In contrast to αs2-casein, αs1-casein has a flexible, loose tertiary structure, the ‘random coil’ conformation, due to its lack of disulphide bonds, high number of proline residues and the presence of hydrophobic amino acids comprising nearly 50% of the protein [14,15]. Consequently, most antibodies binding to αs1-casein bind ‘linear’ epitopes, with a minority directed at conformational epitopes [16,17].
Although β-lactoglobulin has been reported to be the major allergenic protein in cow’s milk [18], recent studies have highlighted the importance of caseins in terms of frequency and intensity of IgE binding [19,20]. In agreement with these more recent studies, few patients in our study showed specific IgE-binding to whey proteins. Moreover, caseins seem to be the main allergen in older children and adults allergic to CM [21,22]. Indeed, Sicherer and Sampson reported that children with persistent CMA possessed significantly higher casein-specific IgE levels than younger children who outgrew their CMA [8].

Kohno et al. studied specific IgE binding to native and denatured α-casein in children with CMA and found no differences in the percentage of binding activity [23]. However, when native and denatured α-casein were compared by gel electrophoresis, they saw no differences between the two forms of protein, raising some questions as to the adequacy of denaturation. In the present study we not only found differences in IgE binding to linearized and native α-casein, but also showed different electrophoretic patterns of the modified proteins on native gels. In contrast to our study, none of the denaturing protocols by Kohno et al. included DTT, which not only breaks disulphide bonds in αs2-casein dimers, but also unfolds the random coil conformation of α-casein at a high concentration.

The six selected peptide epitopes (two from αs1-casein (A-I and A-II) and four from β-casein) did not coincide with the major phosphorylation sites in αs1-casein (aa

Fig. 3. Specific IgE binding to six selected decapeptides from αs1-casein and β-casein in 10 children (patients A to J) who achieved clinical tolerance (a) and 10 patients (patients K to T) with persistent CMA (b) using SPOTs membrane. In all patients serum was obtained at the time of clinical reactivity. For αs1-casein: spot A-I corresponds to amino acids in position 69–78 and spot A-II corresponds to amino acids in position 175–192. For β-casein: spot B-I corresponds to amino acids in position 151–160, spot B-II corresponds to amino acids in position 167–176, spot B-III corresponds to amino acids in position 177–188 and spot B-IV corresponds to amino acids in position 193–202.
Fig. 3. continued.

Table 1. IgE binding to specific decapeptides in patients with persistent cow’s milk allergy, patients who became clinically tolerant and non-milk-allergic subjects. In all patients serum was obtained at the time of clinical reactivity. Sera of tolerant patients were used in 10-fold higher concentration.

<table>
<thead>
<tr>
<th>Protein Decapeptide</th>
<th>$\alpha_{\text{S1}}$-casein</th>
<th>$\alpha_{\text{S2}}$-casein</th>
<th>$\beta$-casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of persistent CMA patients showing strong (weak) binding.</td>
<td>60 (0)</td>
<td>80 (0)</td>
<td>40 (10)</td>
</tr>
<tr>
<td>Percentage of tolerant patients showing strong (weak) binding.</td>
<td>0 (0)</td>
<td>20 (50)</td>
<td>50 (50)</td>
</tr>
<tr>
<td>Percentage of non-milk-allergic individuals showing strong (weak) binding.</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

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66–70) and β-casein (aa 17–21), which due to their sequence homology have been postulated to be responsible for the cross-reactivity observed among αs1-casein, αs2-casein and β-casein [24]. None of the children who outgrew CMA recognized peptide A-I and only 20% showed binding to peptide A-II, utilizing 10-fold more concentrated sera. In contrast 60% of the children with persistent CMA had strong binding to A-I and 80% to A-II, respectively.

If certain linear epitopes account for the allergenic property of cow’s milk, industrial cooking and digestive processes may facilitate exposure to allergenic epitopes and contribute to the increased allergen-specific IgE production. This in turn could provoke a local inflammatory response that would enhance mucosal permeability and absorption of milk allergens [25]. This would result in increased CM-specific IgE synthesis and might explain why patients with persistent CMA show higher levels of specific IgE to milk proteins [8].

In our studies we have been able to demonstrate a relationship between specific linear epitopes and the persistence of CMA. Identification of distinct linear epitopes from αs1-casein, which were recognized predominately by children with persistent allergy, may provide a useful tool for predicting the natural history of CMA in milk-allergic children.

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