

# IgE and IgG Binding Epitopes on $\alpha$ -Lactalbumin and $\beta$ -Lactoglobulin in Cow's Milk Allergy

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## Key Words

Cow's milk allergy ·  $\beta$ -Lactoglobulin ·  $\alpha$ -Lactalbumin · B cell epitope · Oral tolerance

## Abstract

**Background:** Cow's milk is one of the most common causes of food allergy in the first years of life. We recently defined IgE and IgG binding epitopes for  $\alpha$ <sub>s1</sub>-casein, a major cow's milk allergen, and found an association between recognition of certain epitopes and clinical symptoms of cow's milk allergy (CMA). Since  $\alpha$ -lactalbumin (ALA) and  $\beta$ -lactoglobulin (BLG) are suspected to be significant allergens in cow's milk, we sought to determine the structure of sequential epitopes recognized by IgE antibodies to these proteins. We further sought to assess the pattern of epitope recognition in association with the clinical outcome of CMA. **Methods:** According to the known amino acid sequence of ALA and BLG, 57 and 77 overlapping decapeptides (offset by two amino acids), respectively, were synthesized on a cellulose derivatized membrane. Sera from 11 patients 4–18 years of age with persistent CMA (IgE to cow's milk > 100 kU<sub>A</sub>/l) were used to identify IgE binding epitopes. In addition, 8 patients < 3 years of age and likely to outgrow their milk allergy (IgE to cow's milk < 30 kU<sub>A</sub>/l) were used to investigate the differences in epitope recognition between patients with 'persistent' and those with 'transient' CMA. Seven pa-

tients 4–18 years of age were used for assessing the IgG binding regions. **Results:** In patients with persistent allergy, four IgE binding and three IgG binding regions were identified on ALA, and seven IgE and six IgG binding epitopes were detected on BLG. The younger patients that are likely to outgrow their allergy recognized only three of these IgE binding epitopes on BLG and none on ALA. **Conclusions:** The presence of IgE antibodies to multiple linear allergenic epitopes may be a marker of persistent CMA. The usefulness of IgE binding to distinct epitopes on whey proteins in defining the patients that would have a lifelong CMA needs to be investigated in further studies.

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## Introduction

Cow's milk is one of the most common causes of food allergy in the first years of life, with approximately 2–2.5% of newborns experiencing allergic reactions to cow's milk during this time [1, 2]. The majority of children 'out-grow' their cow's milk allergy (CMA; become clinically tolerant) by 3–4 years of age. However, 15% of infants with IgE-mediated CMA retain their sensitivity into the second decade [3]. Cow's milk proteins have been well characterized. However, the relative allergenicity of each protein remains unclear, although data from more recent

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studies [4, 5] have emphasized the importance of the caseins as major milk allergens, but showed also significant reactivity to the whey proteins  $\alpha$ -lactalbumin (ALA) and  $\beta$ -lactoglobulin (BLG).

ALA is a monomeric globular protein of 123-amino acid residues with a molecular weight of 14.4 kD and four disulfide bridges [6]. Marked homology is found between bovine and human ALA, with 74% of the residues identical and another 6% showing chemical similarity. It possesses a high-affinity binding site for calcium, which stabilizes its secondary structure. Its hydrophilic profile suggests that the major antigenic site is located on peptide corresponding to amino acids (AA) 5–18, which corresponds to the homologous sequence 124–134 on the BLG molecule [6].

BLG occurs naturally in the form of a 36-kD dimer with each subunit corresponding to a 162-residue polypeptide [6]. It possesses two disulfide bridges and one free cysteine. It has no homologous counterpart in human milk. The relative resistance of BLG to acid hydrolysis and to proteases allows some of the protein to remain intact after digestion, increasing the probability of absorption of both intact BLG and digested peptides. BLG, together with several allergens of animal origin, belongs to the lipocalin family, which share a similar tertiary (beta-barrel) structure and high affinity for hydrophobic ligands, a characteristic that makes them potentially highly allergenic. The hydrophilic profile of the protein suggests the antigenicity of sequence corresponding to AA 124–134 [6].

Previous studies that utilized digested peptide fragments or synthetic peptides have reported different findings with respect to the major sequential IgE binding regions on ALA [7, 8] and BLG [7, 9–14]. In this study, we mapped sequential (linear) epitopes recognized by IgE and IgG antibodies using synthetic, overlapping decapeptides immobilized on a derivatized cellulose membrane and sera from patients with persistent CMA and those who are likely to outgrow their allergy. Furthermore, we investigated the possibility that a certain pattern of epitope recognition would be associated with the clinical outcome of CMA, as recently shown for  $\alpha_{s1}$ -casein [15].

## Material and Methods

### Subjects

The patient population consisted of 21 children with IgE-mediated CMA. The first group included 13 patients (9 males, 4 females) 4–18 years of age, with a median age of 8 years, and milk-specific IgE antibodies more than 100 kU<sub>A</sub>/l measured by the CAP

System FEIA<sup>TM</sup> (Pharmacia Diagnostics; Uppsala, Sweden). Based on the clinical history, 5 of the patients had atopic dermatitis and 2 had urticaria, 3 experienced both cutaneous and gastrointestinal symptoms (vomiting and/or abdominal pain and hives/pruritus) and 3 reacted with anaphylaxis to cow's milk. During follow-up, 6 of these patients with presumed persistent CMA were shown by challenge (n = 2), or accidental ingestion (n = 4) to still have CMA at the median age of 12 years (range 7–12 years). The other 7 patients had a convincing history of severe immediate reaction to cow's milk and median level of milk-specific IgE still above 100 kU<sub>A</sub>/l (range 56 to >100 kU<sub>A</sub>/l), showing a 95% predictive value of positive challenge to cow's milk [16]. Of these 13 patients, 11 were used to identify the IgE binding epitopes and 7 patients to detect IgG binding epitopes. Of the 11 patients tested for binding of IgE epitopes, 4 patients had levels of specific IgE antibodies against ALA and 4 against BLG over 100 kU<sub>A</sub>/l.

The second group included 8 patients with CMA (6 males, 2 females) 1–3 years of age (median 2 years) with milk-specific IgE levels ranging from 10.8 to 27.6 kU<sub>A</sub>/l. Their sera were pooled for identifying IgE binding sites. These children showed slow and progressively declining levels of cow's milk-specific IgE, and were therefore considered likely to outgrow their CMA. Of them, 1 had atopic dermatitis, 4 had gastrointestinal symptoms (colic, protracted vomiting and diarrhea), 2 experienced both cutaneous and gastrointestinal symptoms (prolonged colic), and 1 experienced anaphylaxis.

Control sera were obtained from 5 atopic, non-milk-allergic individuals with atopic dermatitis and IgE-mediated allergy to other foods (i.e. tomato, shellfish, peanuts and nuts), aged 5–11 years. They had negative skin prick tests to milk and/or milk-specific IgE antibodies less than 0.35 kU<sub>A</sub>/l, and clinical tolerance to cow's milk.

Informed consent was obtained, and the study was approved by the Institutional Review Board of Mount Sinai School of Medicine.

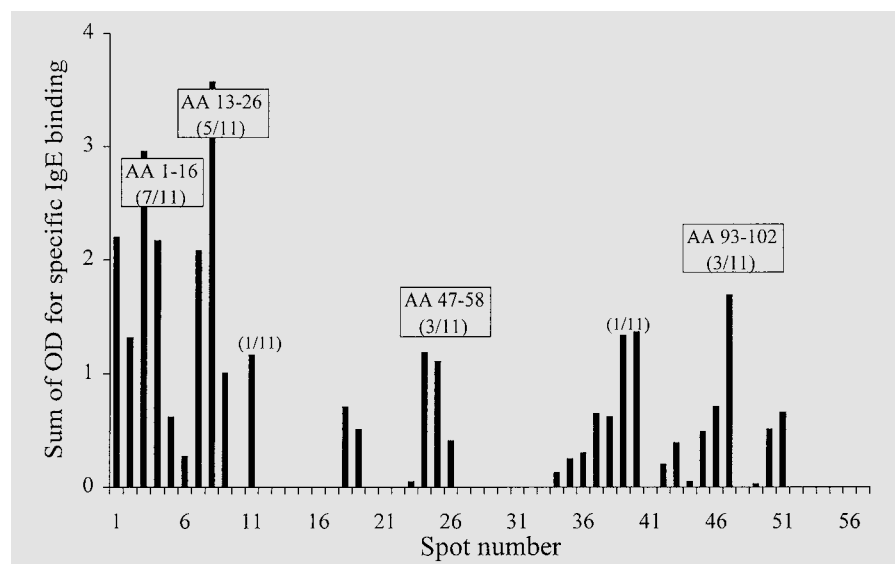
### Peptide Synthesis

SPOTs<sup>TM</sup> membranes (Genosys Biotechnologies, Woodlands, Tex., USA), derivatized cellulose membranes, were used to generate multiple decapeptides (77 in BLG and 57 in ALA), overlapping by eight amino acids and covering the entire sequences of the two proteins. Individual peptides were synthesized on the membrane by the 9-fluorenyl-methoxycarbonyl (Fmoc) method according to the manufacturer's instruction. Fmoc-amino acid derivatives were dissolved in 1-methyl-2-pyrrolidone and loaded on marked spots on the membrane. Coupling reactions were followed by acetylation with 4% acetic anhydride in N,N-dimethylformamide. The membrane was then stained with bromophenol blue to identify the location of the free amino groups. Cycles of coupling, blocking and deprotection were repeated until the peptides of the desired length were synthesized. After the addition of the last amino acid in the peptide, the amino acid side chains were N-terminally acetylated and 'deprotected' using dichloromethane/trifluoroacetic acid/tri-isobutylsilane. Membranes were used for IgE and IgG binding assay as indicated below.

### Identification of IgE and IgG Epitopes

The membranes were blocked overnight with PBS containing 0.01% Tween 20 and 1.5% human serum albumin for detection of IgE epitopes on ALA and BLG, and with 1.5% human serum albumin together with 5% rabbit and additional 2% normal human serum for detection of IgG epitopes. For detection of antibody binding to linear epitopes, individual and pooled sera were diluted 1:10 for IgE and 1:20 for IgG. Serum samples were incubated with the

**Fig. 1.** Cumulative OD scores of IgE antibodies for each of the synthetic decapeptides of ALA. According to the known AA sequence, 57 decapeptides, overlapping by 8 AAs, were synthesized on a derivatized cellulose membrane. Sera from 11 milk-allergic children over the age of 4 years with high levels of specific serum IgE were used to identify IgE binding epitopes. The boxes above the cumulative OD bars indicate the AA sequence corresponding to the allergenic epitope, and the number of patients recognizing each epitope. Control subjects had no binding to any linear epitopes.



membranes overnight at 4°C. IgE and IgG antibody binding was detected utilizing an immunoenzymatic method with biotinylated goat anti-human IgE and rabbit anti-human IgG, and peroxidase streptavidin conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA). Dilutions of sera and secondary antibody were based on preliminary experiments in which the intensity of the background was assessed separately for each protein to achieve a minimum of background that would not interfere with the interpretation of the results. The membranes were subsequently developed with a chemiluminescent detection system (Amersham, Arlington Heights, Ill., USA).

After developing the x-ray film, optical density (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 peptide spot and the OD of background. Each of the decapeptides was assigned a 'cumulative score' that represented the sum of the ODs from every patient in each group. The cumulative scores and individual scores from each patient were used to identify particular regions on the protein that were recognized by patients as a group and by individual patients. True binding was defined as OD > 0.3 for pooled sera and > 0.2 for individual sera. An epitope was defined as a protein sequence recognized by at least 25% of the patients.

## Results

### *IgE Binding Epitopes of ALA*

Using sera from the 11 older patients with CMA, four IgE binding sites were identified on ALA (fig. 1). IgE antibodies from 8 of 11 patients showed binding to at least one linear epitope. The number of patients recognizing each of the epitopes is shown in figure 1. Interestingly, of those four patients that were known to have high levels of

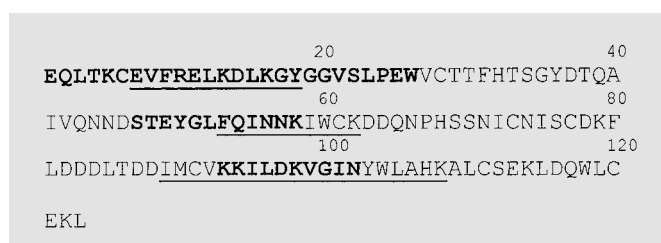


**Fig. 2.** Amino acid sequence of ALA. IgE binding (allergenic) epitopes are shown in bold. IgG binding (antigenic) epitopes are underlined.

IgE antibodies specifically to ALA (>100 kU<sub>A</sub>/l), only two showed binding to linear epitopes, reflecting the predominance of conformational epitopes of ALA (data not shown). The complete AA sequence of the protein with IgE binding epitopes depicted is shown in figure 2. None of the linear epitopes were recognized by the younger patients or by the control subjects (data not shown).

### *IgG Binding Epitopes of ALA*

Three IgG binding regions were identified on ALA using sera from 7 older patients with CMA (fig. 2, 3). The number of patients who possess antibody binding to these epitopes is shown in figure 3. Of the 5 controls, 3 recognized the first epitope, and 2 showed binding to the third IgG binding epitope (fig. 3).

**Fig. 3.** Cumulative OD scores of IgG antibodies for each of the synthetic decapeptides of ALA. Sera from 7 milk-allergic children over the age of 4 years were used to identify IgG binding epitopes. The boxes above the cumulative OD bars indicate the AA sequence corresponding to the antigenic epitope, and the number of patients (P) and controls (C) recognizing each epitope.

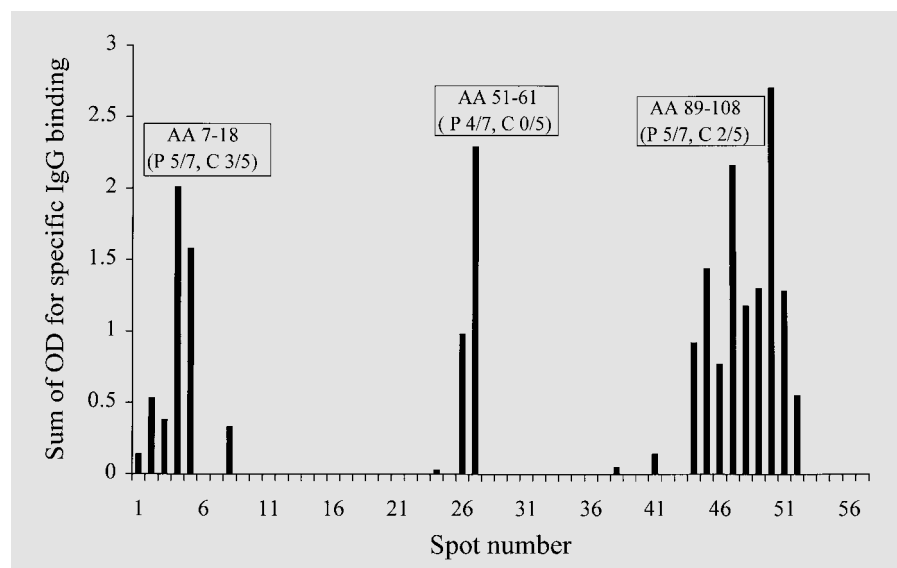


**Fig. 4.** Cumulative OD scores of IgE antibodies for each of the synthetic decapeptides of BLG. According to the known AA sequence, 77 decapeptides, overlapping by 8 AAs, were synthesized on a cellulose-derivatized membrane. Sera from 11 milk-allergic children over the age of 4 years with high levels of specific serum IgE (**a**), and pooled sera from 8 patients with CMA who were less than 3 years of age with low levels of milk-specific IgE (**b**) were used to identify IgE binding epitopes. The boxes above the cumulative OD bars indicate the AA sequence corresponding to the allergenic epitope, and the number of patients recognizing each epitope. Control subjects had no binding to any linear epitopes.

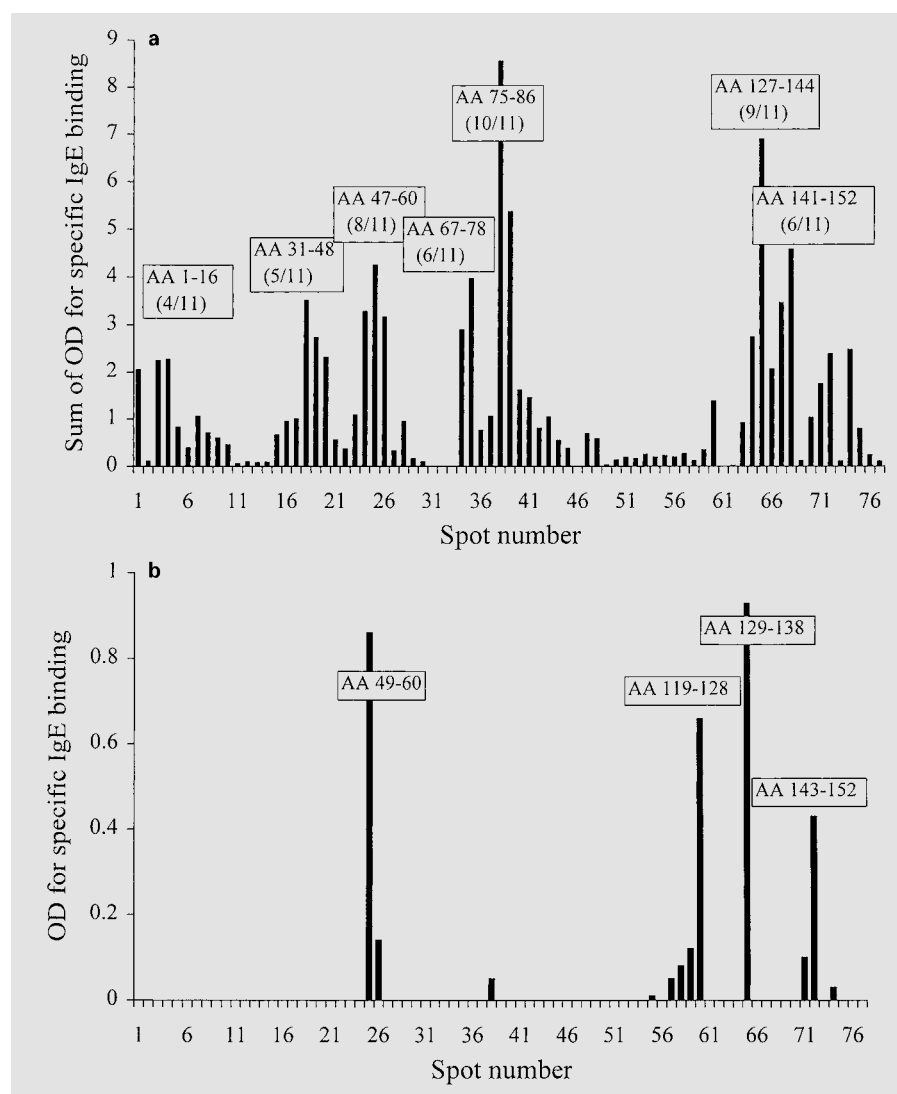


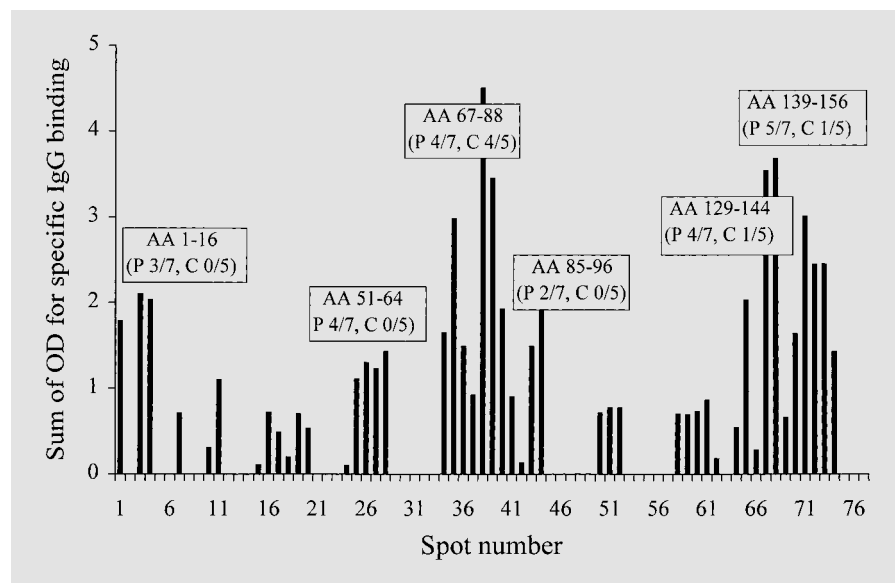
**Fig. 5.** Amino acid sequence of BLG. IgE binding epitopes are shown in bold. IgG binding epitopes are underlined.

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                20                                40
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLR
                60                                80
VYVEELKPTPEGDLEILLQKWENDECAQKKIIAEKTKIPA
                100                               120
VFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQ
                140                               160
CLVRTFEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQC
HI

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**Fig. 6.** Cumulative OD scores of IgG antibodies for each of the synthetic decapeptides of BLG. Sera from 7 milk-allergic children over the age of 3 years with high levels of specific serum IgG were used to identify IgG binding epitopes. The boxes above the cumulative OD bars indicate the AA sequence corresponding to the antigenic epitope, and the number of patients (P) and controls (C) recognizing each epitope.



#### *IgE Binding Epitopes of BLG*

Using sera from 11 older patients, we identified seven IgE binding regions on BLG (fig. 4a). In comparison to ALA, all patients recognized at least one epitope on BLG. The number of patients who had antibody recognizing each of these epitopes is shown in figure 4a. The complete AA sequence of the protein with IgE binding epitopes is depicted in figure 5. The younger patients with a low level of IgE to cow's milk and who were likely to outgrow their CMA recognized only three of the seven identified epitopes (fig. 4b). In addition, the region corresponding to AA 119–128, which showed weak binding with sera from the older patients, and was therefore not defined as a major epitope (fig. 4a), proved to show intensive binding among the younger patients (fig. 4b). Sera from control subjects showed no binding to any of the linear peptides.

#### *IgG Binding Epitopes of BLG*

Using the sera from 7 older patients, six IgG binding regions were identified on BLG (fig. 5, 6). The number of

patients who had antibody binding to these epitopes is shown in figure 6. Of the 5 control subjects, 4 recognized the third epitope, and 1 showed binding to the fifth and 1 to the sixth epitope (fig. 6). Additionally, 2 control subjects recognized the region corresponding to AA 35–46, and 1 bound to AA 115–126, which were also IgG binding sites for 2 of the patients, but not considered as major IgG binding epitopes (data not shown).

#### **Discussion**

Cow's milk is a significant cause of food allergy in early childhood. In the present study, we identified several linear IgE binding epitopes on BLG and fewer on ALA in milk-allergic patients by using synthetic peptides, and suggest that binding to some of these epitopes may be associated with the persistence of CMA. We also report for the first time the locations of IgG binding epitopes on these whey proteins.

To our knowledge there are only two studies reporting IgE binding epitopes on ALA performed with human sera from CMA patients [7, 8]. In the study by Adams et al. [7], the fragment corresponding to AA 5–18 was shown by RAST inhibition to exhibit IgE binding capacity. In another study using trypsin-digested ALA, a sequence corresponding to AA 17–58 and larger peptides sharing this sequence were most strongly and frequently recognized [8], and binding to smaller peptides corresponding to sequences AA 6–10:S-S:115–123 and AA 109–123 was also reported. It has been suggested that because the majority of patients (60%) responded to native ALA and larger peptides, the conformational epitopes are 'more important' [8]. Whether or not these epitopes are clinically relevant, our findings of infrequent binding to linear peptides of ALA in the patients with high levels of specific IgE to ALA support the high frequency of binding to conformational epitopes. The IgE binding epitopes observed in the present study (1–18, 13–26, 47–58 and 93–102) coincide with allergenic epitopes reported in previous studies, although exact comparisons are difficult to perform because the tryptic cleavage of ALA yields considerably longer residues. However, the carboxyl terminal end of the protein (109–123) was not recognized by IgE from our patients. Furthermore, the younger patients with a lower level of IgE antibodies and who were likely to outgrow their allergy did not recognize any linear epitopes on ALA.

It has been shown that tryptic cleavage of BLG largely reduces the immunoreactivity (<50%) for most patient sera (75%) [12]. The hydrophilicity profile of the protein suggests that the antigenic site is at AA 124–134, which is on the surface of the protein and described as a major allergenic site in our study and those of other investigators [7, 9, 13]. AA 97–108 was described by Ball et al. [10] as a major linear epitope, which corresponded to a major epitope (AA 95–113) also reported by Heinzmann et al. [14], but not confirmed in our study or that of Williams et al. [13]. Selo et al. [12] reported the tryptic fragments AA 1–8, 25–40, 41–60, 102–124 and 149–162 as major IgE binding epitopes in a population of milk-allergic patients whose ages were not reported. These findings are in good agreement with our results, although the most frequently bound epitopes in our study, AA 75–86 and 127–146, were not reported by them. The first of these, AA 75–86, was recognized only by the patients with persistent CMA, which might explain why it was such an important epitope in our study but was not reported previously.

IgG binding epitopes on ALA or BLG have not been mapped in cow's milk-allergic patients. In the present

study, we identified three IgG binding sequences on ALA which were AA 7–18, 53–62, 89–108, in partial agreement with earlier findings using rabbit antisera [17], and those predicted by the hydrophilicity profile [8]. The IgG binding regions identified in our study colocalized with the IgE binding epitopes in milk-allergic patients, except for the sequence AA 13–26. This suggests that allergenic sites of bovine ALA do not totally correspond to antigenic determinants, which was also reported by Maynard et al. [8].

Studies investigating BLG-specific IgG binding epitopes by ELISA, using both native and pepsin-digested products, have suggested that IgG antibodies from milk-allergic patients show a marked preference for conformational epitopes whereas normal individuals recognize both conformational and sequential ones [18]. While the presence of IgE antibodies to foods have been associated with clinical hypersensitivity, the IgG antibody formation to food is physiologic. IgG antibodies are present at birth, and the subsequent increase in specific IgG antibodies depends upon the type of feeding, indicating the importance of intestinal exposure to food allergens [19]. Early exposure (feeding cow's milk during the first 3 days of life) stimulated higher levels of IgG antibody to cow's milk protein that persisted through 2 years of life, when compared to avoidance of early exposure [20]. The pathogenic role of an IgG response in CMA has not been established and studies reporting comparisons of serum milk-specific IgG concentrations between CMA patients and controls are conflicting [21–23]. However, significantly higher levels of IgG antibodies against certain milk proteins have been found in children with persistent CMA [21, 24] and adult milk-intolerant patients [25]. In the present study, a close relationship appears to exist between the specificity of IgE and IgG antibodies in individuals with persistent CMA, strongly supporting the concept of differences in the antigen absorption and/or handling in the gastrointestinal mucosa in these patients.

In the present study, the IgG binding sequences generally colocalized with the IgE binding epitopes, except for three short regions (AA 1–6, AA 19–26 and AA 47–52) on ALA, and one extended region (AA 31–50) on BLG, which appeared to be exclusive allergenic epitopes. Although there is a possibility that this difference could be explained by lower affinity of IgG antibodies to these epitopes, this finding suggests that epitopes responsible for IgE synthesis differ slightly from those promoting IgG production. Lack of specific IgG to these epitopes could make these epitopes more accessible for IgE recognition, possibly contributing to the persistence of CMA.

The complete AA sequence of bovine ALA shows extensive homology with human ALA; 74% of the residues are identical and another 6% are chemically similar. However, despite this great similarity, bovine ALA has been reported as one of the major allergenic proteins in cow's milk [26]. This is not consistent with our findings here or in a previous study [5]. Interestingly, the sequence AA 7–22 is one of two regions showing the highest degree of IgE binding. Simultaneously, this part of bovine ALA is characterized by its low degree of sequence homology to human ALA, and may therefore play an important role in its allergenicity. In comparison, BLG is absent in human milk, but has extensive homology to several human and animal proteins of the lipocalin family, some of which are found in endogenous body fluids in humans. This does not seem to exclude the possibility of an allergenic response to bovine BLG.

Identification of the major IgE binding epitopes on milk proteins, as in other food proteins, is important in the development of immunotherapeutic interventions for food allergies. In a previous study, we found that the epitope-specific IgE to another milk protein,  $\alpha_{s1}$ -casein was present at (or near) the time of diagnosis [13]. Therefore, the presence of IgE antibodies to these 'informative' epi-

topes in patients' sera may be useful in distinguishing which children will develop lifelong CMA. However, there appears to be little difference in ALA- or BLG-IgG antibody specificity between CMA patients and control subjects, indicating that only the IgE antibody specificity to these proteins affects clinical reactivity.

In conclusion, IgE binding to sequential peptides of ALA is limited and less extensive than the binding to linearized BLG, suggesting that patients with CMA also recognize linear allergenic epitopes on BLG whereas allergenic regions on ALA are conformational. Furthermore, the presence of IgE antibodies to multiple linear allergenic epitopes may serve as a marker of persistent CMA. The usefulness of the IgE epitopes in whey proteins that were not recognized by the patients likely to outgrow their CMA and defining the patients that would have a lifelong CMA need to be investigated in further studies.

## Acknowledgments

This work was supported by grants AI 44236 and AI 24439 from the National Institute of Allergy and Infectious Diseases, M01 RR00052 from the Division of Research Resources, National Institutes of Health, and the Bunning Family Fund.

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