

# Identification of IgE- and IgG-binding epitopes on $\alpha_{s1}$ -casein: Differences in patients with persistent and transient cow's milk allergy

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**Background:** Cow's milk allergy (CMA) affects 2.5% of children less than 2 years of age, but about 80% become clinically tolerant within the first 3 years of life. Casein is one of the major allergens responsible for CMA and seems to play an important role in persistent allergy. Previous studies on egg allergy suggested that linear epitopes are associated with long-lasting food allergy.

**Objective:** The aim of the study was to identify IgE- and IgG-binding epitopes on  $\alpha_{s1}$ -casein and to determine whether the patterns of epitope recognition are associated with the natural history of CMA.

**Methods:** According to the known amino acid (AA) sequence, 96 overlapping decapeptides representing the entire length of  $\alpha_{s1}$ -casein were synthesized on a cellulose-derived membrane. Sera from 24 children with milk allergy were used to identify IgE- and IgG-binding epitopes.

**Results:** Six major and 3 minor IgE-binding, as well as 5 major and 1 minor IgG-binding, regions on  $\alpha_{s1}$ -casein were identified. Two IgE-binding regions (AA 69-78 and AA 173-194) were recognized by the majority of patients over 9 years of age with persistent allergy (67% and 100%, respectively) but by none of the children less than 3 years of age who are likely to outgrow CMA. No differences in IgG binding between the groups were observed.

**Conclusion:** There appears to be a difference in epitope recognition between patients with different natural histories of CMA. Screening for IgE antibodies to these epitopes may be useful in identifying children who will have persistent milk hypersensitivity. (*J Allergy Clin Immunol* 2001;107:379-83.)

**Key words:** Cow's milk allergy, tolerance, epitope, conformational, linear,  $\alpha_{s1}$ -casein, spot membrane

Cow's milk allergy (CMA) affects approximately 2.5% of children less than 2 years of age,<sup>1-4</sup> with slightly over one half of the reactions being IgE mediated.<sup>1</sup> Casein is one of the major allergens responsible for

## Abbreviations used

AA: Amino acid

CMA: Cow's milk allergy

CMA. It is the main component in cow's milk, constituting 80% of the total protein. Cow's milk casein is comprised of 4 proteins,  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -, and  $\kappa$ -casein. These proteins have little primary structure homology.<sup>5</sup>  $\alpha_{s1}$ -Casein is the most abundant protein in cow's milk, constituting 34% of total milk proteins. It is a single-chain phosphoprotein of 199 amino acids (AAs) characterized by a high content of proline residues distributed throughout the molecule.<sup>6</sup>  $\alpha_{s1}$ -Casein has nearly 70% unordered structure, with only a small amount of secondary structure, such as  $\alpha$ -helix or  $\beta$ -sheets.<sup>7,8</sup> In addition, it lacks disulfide bonds, resulting in a reduction of tertiary interactions.<sup>8</sup> Thus far 2 groups have attempted to identify IgE- and IgG-binding regions in  $\alpha_{s1}$ -casein in human subjects, with different results. The first group used overlapping synthetic peptides in combination with digestion fragments and sera from 9 patients with CMA (age, 0-6 years; mean age, 3 years). A single immunodominant IgE-binding region at the carboxyl terminal (AA 181-199) was identified.<sup>9</sup> The second group used overlapping peptides synthesized on polyethylene pins and sera from 15 patients with CMA (age, 4 months-30 years; median age, 21 months) to screen for IgE- and IgG-binding regions by ELISA.<sup>10</sup> They identified 7 IgE-binding epitopes with 3 immunodominant epitopes (regions 19-30, 93-98, and 141-150). These 3 epitopes were characterized by a high content of nonpolar and aromatic AAs. They found no essential differences in the epitope specificity between IgG and IgE.

About 80% to 85% of children with CMA outgrow their allergy (become clinically tolerant) by 3 years of age, whereas the remaining 15% to 20% do not lose their clinical reactivity.<sup>1</sup> The immunologic mechanism of this phenomenon is still unknown. There is evidence suggesting that casein plays an important role in persistent allergy. IgE antibodies binding to casein appeared to be dominant in older children and adults with CMA.<sup>11,12</sup> Children who have persistent CMA have significantly elevated levels of casein-specific IgE antibodies compared with younger children.<sup>13</sup>

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The binding of IgE antibodies to linear, as opposed to conformational, epitopes has been associated with the natural history of egg allergy in previous studies of patients allergic to eggs. These results suggested that protracted food hypersensitivity is associated with the development of significant quantities of IgE antibodies to linear epitopes, whereas transient food hypersensitivity is associated with IgE antibodies to conformational epitopes.<sup>14</sup> The relationship between IgE antibodies to linear epitopes and persistent hypersensitivity in children with CMA has not been studied.

The advances in peptide synthesis technology provide a more precise identification of linear epitopes on proteins of interest.<sup>15,16</sup> By synthesizing multiple overlapping peptides onto a derivatized cellulose membrane, these peptide-bound membranes can be used to identify particular linear areas on the protein recognized by patients' antibodies.<sup>17</sup>

In this study overlapping synthetic peptides were used to identify major IgE- and IgG-binding epitopes of  $\alpha_{s1}$ -casein in patients with CMA. Identification of the immunodominant epitopes of caseins may lead to a better understanding of the mechanisms responsible for sensitization to these major milk allergens. In addition, we have identified qualitative differences in the pattern of epitope recognition by IgE antibodies between older patients with persistent CMA and younger patients who are likely to outgrow their allergy.

## METHODS

### Patient population

Sera from 24 patients (median age, 8 years; range, 1-17 years; 19 male and 5 female patients) with IgE-mediated CMA were used to identify IgE- and IgG-binding epitopes. Milk-specific IgE antibodies ranged from 0.8 to more than 100 kU<sub>A</sub>/L, as measured by the CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). To identify differences in the IgE- and IgG-binding pattern among the patients with CMA, 2 subgroups were chosen. Group A consisted of 9 patients over 9 years of age (median age, 12 years; range, 9-17 years; 7 male and 2 female patients) with persistent CMA. All of them had milk-specific IgE antibodies from 60 to more than 100 kU<sub>A</sub>/L. Group B consisted of 8 patients less than 3 years of age (median age, 2 years; range, 1-3 years; 6 male and 2 female patients) who were likely to outgrow their milk allergy. Their levels of milk-specific IgE antibodies were less than 30 kU<sub>A</sub>/L (median, 18.6 kU<sub>A</sub>/L). Seven patients from 3 to 9 years of age were not included in this comparison. Control sera were obtained from 10 atopic individuals without milk allergy with negative skin prick test responses to milk, milk-specific IgE antibodies, or both of less than 0.35 kU<sub>A</sub>/L and clinical tolerance to cow's milk. Five of the control patients were 9 years or older (median age, 10 years; range, 9-19 years; 3 male and 2 female patients), and 5 control subjects were 3 years of age or younger (median age, 2 years; range, 1.25-3 years; 3 male and 2 female patients). Informed consent was obtained, and the study was approved by the institutional review board.

### Preparation of SPOTs membrane of overlapping peptides

SPOTs membrane (Genosys Biotechnologies, Woodlands, Tex), a derivatized cellulose membrane, was used as a support to generate 96 decapeptides for  $\alpha_{s1}$ -casein, overlapping by 8 AAs and cov-

ering the entire sequence of the proteins. Individual peptides were synthesized on the membranes by the 9-fluorenyl-methoxycarbonyl method, according to the manufacturer's instructions. 9-Fluorenyl-methoxycarbonyl AA derivatives were dissolved in 1-methyl-2-pyrrolidone and applied 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 adding the tenth AA to the synthesized peptide, the AA side chains were deprotected by using a mixture of dichloromethane/trifluoroacetic acid/trisobutylsilane. Membranes were used for IgE- and IgG-binding assays, as indicated below.

### Probing the SPOTs membrane with patient serum

The membrane was blocked overnight with PBS containing 0.1% Tween-20 and 1% human serum albumin. Diluted individual patients' sera (1:50 for IgE and 1:200 for IgG binding) were incubated with the membranes overnight at 4°C. IgE and IgG antibody binding was detected by using an immunoenzymatic method with goat anti-human IgE or rabbit anti-human IgG and peroxidase streptavidin conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md). The membranes were developed with a chemiluminescent-avidin-horseradish peroxidase detection system (Amersham, Arlington Heights, Ill).

After developing the x-ray film, the OD of each individual peptide spot was measured by means of reflection densitometry. The OD of each peptide spot was recorded as the difference between the OD of the peptide spot and background. Each of the decapeptides was assigned a cumulative score that represented the sum of the ODs from all patients in each group, as previously described.<sup>13</sup> 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. Major IgE- or IgG-binding regions are defined as regions that are bound by IgE antibodies from at least 50% of patients with CMA. Cutoff level for binding was an OD of 0.2 for individual binding. This cutoff level was chosen because it provided a clear distinction between specific and nonspecific binding.

## RESULTS

### IgE-binding epitopes of $\alpha_{s1}$ -casein

Using cumulative ODs for the total study population, 6 major and 3 minor IgE-binding regions on  $\alpha_{s1}$ -casein were identified (Fig 1). Their locations throughout the protein are shown in Fig 2. Densitometric scanning indicated that the highest cumulative intensity of IgE binding was in regions 17-36, 69-78, 109-120, and 173-194 (Fig 1), recognized by 75%, 46%, 53%, and 63% of the patients, respectively. Regions 69-78 and 109-120 had AA sequence homology, both showing a sequence of X<sub>1</sub>EIVPNSX<sub>2</sub>EX<sub>3</sub>, where X<sub>1</sub> was glutamic acid (E) versus leucine (L), X<sub>2</sub> was valine (V) versus alanine (A), and X<sub>3</sub> was glutamine (Q) versus glutamic acid (E; Fig 2). The median number of IgE-binding regions recognized per individual was 5 (range, 0-9; data not shown). Sera from control patients showed no IgE binding to the peptides (data not shown).

Comparing patients with persistent CMA with the patients from the younger age group who are likely to

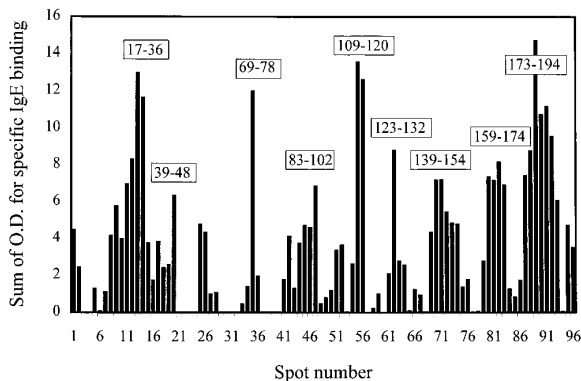


**FIG 1.** Cumulative OD scores for IgE for each of the synthetic decapeptides of  $\alpha_{s1}$ -casein. According to the known AA sequence, 96 overlapping decapeptides were synthesized on a cellulose-derivatized membrane. Sera from 24 children with milk allergy were used to identify IgE-binding epitopes. Numbers on top of bars represent the actual AA.

outgrow their CMA, 7 of the 9 regions were recognized by patients from both groups (Table I). In contrast, 2 IgE-binding regions (AA 69-78 and AA 173-194) were recognized by the majority of patients in the older age group (67% and 100%, respectively) but by none of the patients in the younger age group (Table I). In addition, individual patients from the older age group recognized a greater number of IgE-binding epitopes than individuals in the younger age group (median, 7 vs 2; data not shown).

### Comparison of IgE binding in patients with persistent CMA at different ages

To study the development of IgE antibody epitope specificity over the course of persistent CMA, stored sera from 3 patients with persistent CMA were used to evaluate IgE-binding specificity when patients were young and several years later. The sera used were obtained at the following ages: patient 1 at 2 and 9 years, patient 2 at 3 and 14 years, and patient 3 at 1 and 10 years of age (Fig 3). There was little change in individual IgE epitope recognition patterns over the 7- to 11-year time period, with the minimal development of IgE antibodies to some new epitopes and the minimal decrease or disappearance of previously existing epitope-specific IgE antibodies. Interestingly, at a very young age, all 3 patients had already developed epitope-specific IgE to regions corresponding to AA 69-78 and AA 173-194, which were the epitopes distinguishing between the patients with persistent CMA from those patients who were likely to outgrow CMA. Moreover, the intensity of IgE binding to the 2 regions persisted, increased, or both when they grew older. Patients in the younger age group who were likely to outgrow CMA, as mentioned earlier, did not recognize these 2 regions.

### IgG-binding epitopes of $\alpha_{s1}$ -casein

Using cumulative ODs for the total study population, 5 major and 1 minor IgG-binding regions on  $\alpha_{s1}$ -casein

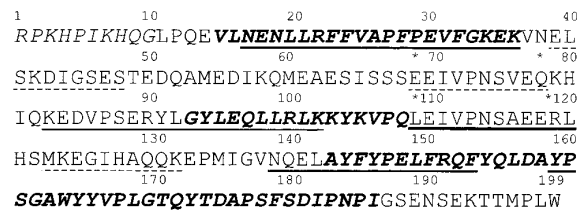


**FIG 2.** AA sequence of  $\alpha_{s1}$ -casein. IgE-binding epitopes are underlined; thick lines represent major epitopes, and broken lines represent the minor ones. IgG-binding epitopes are shown in italics, and the major epitopes are shown in bold. Asterisks depict the location of the 2 epitopes with the following sequence homology: X<sub>1</sub>EIVPNSX<sub>2</sub>EX<sub>3</sub>.

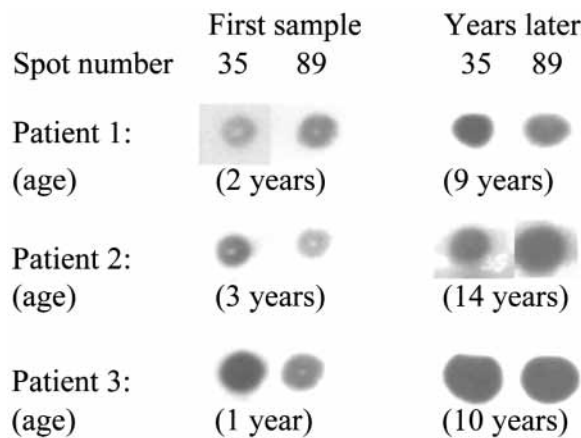


**FIG 3.** Selected part of the Spot membrane showing Spot number 35, corresponding to the third epitope (AA 69-78), and Spot number 89, corresponding to the decapeptide 177-168 in epitope 9 (AA 173-194). Shown is the IgE labeling with sera from 3 patients with persistent CMA at an early phase of the disease and several years later. The sera used were obtained at the following ages: patient 1 at 2 and 9 years, patient 2 at 3 and 14 years, and patient 3 at 1 and 10 years of age.

were identified (Fig 4). Their locations throughout the protein are shown in Fig 2. The most frequently recognized region was AA 15-36, which was recognized by 92% of patients. This region also had the highest cumulative intensity of IgG binding (Fig 4). Region 69-78, which was the IgE-binding epitope and distinguishes the older and the younger age group, was not found to be an IgG-binding epitope. The median number of IgG-binding regions recognized by an individual was 5 (range, 1-6). Six of the control patients had IgG binding to  $\alpha_{s1}$ -casein peptides but with very low intensity. Five of them recognized the region corresponding to AA 15-36, and one recognized the region 143-160.

In contrast to IgE binding, no striking difference existed for IgG binding between the group of older patients with persistent CMA and the group of younger children likely to outgrow their allergy (Table II). Each of the 6 IgG-binding regions was recognized by more than 20% of patients from both groups. The median number of IgG-binding epitopes recognized per individual in both groups was 5.

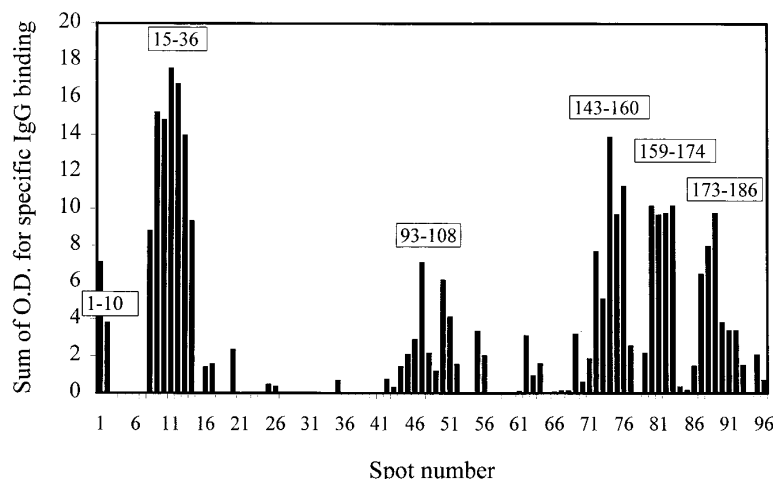


**FIG 4.** Cumulative OD scores for IgG for each of the synthetic decapeptides of  $\alpha_{s1}$ -casein. According to the known AA sequence, 96 overlapping decapeptides were synthesized on a cellulose-derivatized membrane. Sera from 24 children with milk allergy were used to identify IgG-binding epitopes. Numbers on top of bars represent the actual AA.

**TABLE I.** Comparison of patients with persistent CMA (>9 years, n = 9) and patients from the younger age group who are likely to outgrow their allergy (<3 years, n = 8)

| Epitope | 1     | 2     | 3     | 4      | 5       | 6       | 7       | 8       | 9       |
|---------|-------|-------|-------|--------|---------|---------|---------|---------|---------|
| AA      | 17-36 | 39-48 | 69-78 | 93-102 | 109-120 | 123-132 | 139-154 | 159-174 | 173-194 |
| <3 y    | 50%   | 13%   | 0%    | 13%    | 13%     | 13%     | 25%     | 18%     | 0%      |
| >9 y    | 89%   | 44%   | 67%   | 67%    | 89%     | 56%     | 56%     | 56%     | 100%    |

Values are percentages of patients in each group showing IgE binding to the different epitopes.

**TABLE II.** Comparison of patients with persistent CMA (>9 years, n = 9) and patients from the younger age group who are likely to outgrow their allergy (<3 years, n = 8)

| Epitope | 1    | 2     | 3      | 4       | 5       | 6       |
|---------|------|-------|--------|---------|---------|---------|
| AA      | 1-10 | 15-36 | 93-108 | 143-160 | 159-174 | 173-186 |
| <3 y    | 50%  | 88%   | 63%    | 88%     | 75%     | 88%     |
| >9 y    | 22%  | 89%   | 56%    | 56%     | 56%     | 78%     |

Values are percentages of patients in each group showing IgG binding to the different epitopes.

## DISCUSSION

Using overlapping decapeptides, we identified 9 IgE- and 6 IgG-binding epitopes for  $\alpha_{s1}$ -casein. Moreover, 2 of the IgE-binding epitopes identified may be useful for predicting the natural history of CMA in individual patients. These 2 epitopes were recognized by almost all of the patients with persistent CMA but by none of the patients who were likely to outgrow the allergy. The first epitope (AA 69-78) is located in the hydrophilic region of  $\alpha_{s1}$ -casein close to the multiphosphorylation site. The second epitope (AA 173-194) is located near the carboxyl terminal of the protein.

Although there have been several reports delineating the epitopes of  $\alpha_{s1}$ -casein, the data in human subjects is limited. Previous studies performed in mice have shown

varying results.<sup>18-21</sup> The carboxyl terminal end of  $\alpha_{s1}$ -casein has been found to be the major allergenic epitope in mice. As indicated above, 2 groups have attempted to identify IgE- and IgG-binding regions of  $\alpha_{s1}$ -casein in human subjects. Our results are in general agreement with those of these 2 studies. The most frequently recognized IgE-binding epitope in our study (region 173-194) was also the single immunodominant epitope identified by Nakajima-Adachi et al.<sup>9</sup> Spuerger et al.<sup>10</sup> detected 3 immunodominant epitopes (region 17-34, 93-102, and 141-148), which we also identified. However, the 2 regions with AA sequence homology (regions 69-78 and 109-120) that we found to be among the most frequently recognized IgE epitopes (67% and 89%, respectively) were not identified in the previous studies. This discrepancy may be due to the differences in the methods used and the study population. The median age in the study population from Spuerger et al was less than 3 years of age. Of interest is that region 69-78 was not recognized by the younger age group in our study either but by 67% of the older children.

With the identification of 2 unique epitopes (AA 69-78 and AA 173-194) on  $\alpha_{s1}$ -casein that were recognized exclusively by most patients with persistent CMA but none of the patients who were likely to outgrow CMA, the question was raised as to whether the epitope-specific IgE to these 2 areas was simply age dependent. We used past and recent sera from 3 patients with persistent

CMA, collected over a 7- to 11-year period, to document the development of epitope-specific IgE during the course of persistent CMA. We demonstrated that all 3 patients with persistent CMA already had IgE antibodies to these 2 epitopes at a very young age. Therefore the development of IgE antibodies against these 2 unique epitopes was not an age-dependent phenomenon.

Interestingly, epitope AA 69-78 was identified as a unique IgE-binding site. This epitope was recognized by IgE antibodies from the majority of the patients in group A but was not recognized by IgG from any of the patients in either group. This difference could be explained by different affinities of IgE and IgG antibodies to this epitope, but it could also indicate that epitopes responsible for allergen-specific IgE synthesis may differ from those promoting IgG production. There is evidence suggesting a preferential variable chain use in IgE synthesis and a direct switching from IgM to IgE production, in addition to the sequential IgM-IgG-IgE switching progression.<sup>22</sup> Lack of specific IgG to this epitope would make this part of the peptide more accessible for IgE recognition, perhaps contributing to the persistence of CMA. However, the absence of IgG binding to this peptide also could be due to the synthetic peptide, lacking a phosphorylated form. Region 64-75 is the multiphosphorylated segment of  $\alpha_{s1}$ -casein consisting of 5 phosphoserine residues. It has been shown in rabbits that dephosphorylated  $\alpha_{s1}$ -casein had less ability than the native form to inhibit reactions between native  $\alpha_{s1}$ -casein and its IgG antibodies.<sup>19</sup> To date, there have been no data in human subjects addressing the effect of dephosphorylation on IgE and IgG binding to  $\alpha_{s1}$ -casein. However, in our study the peptide was recognized by IgE antibodies from the majority of patients, indicating that the binding of IgE to this peptide is not affected by dephosphorylation.

Our findings on the IgE epitope recognition pattern of  $\alpha_{s1}$ -casein support the significance of linear epitopes in persistent food allergy, as initially hypothesized by Cooke and Sampson<sup>14</sup> in the study of ovomucoid. After protein digestion, the previously complex protein structure will be broken down into multiple peptides that possess mainly linear epitopes. These peptides can penetrate the mature gastrointestinal system in immunologically active forms. In the present study a portion of these linear epitopes were recognized predominantly by the older children with persistent allergy. These epitopes may be an interesting marker for the identification of patients who are at risk for persistent CMA, although our findings cannot explain the mechanistic reason of why the group reacting to these epitopes is still allergic in later years.

In conclusion, we determined IgE- and IgG-binding epitopes on  $\alpha_{s1}$ -casein. In addition, we identified 2 unique epitopes that are recognized only by IgE antibodies from patients with persistent CMA. Screening for antibodies to these epitopes may be useful in identifying

children who will not outgrow their milk allergy and therefore would be candidates for immunotherapy when it becomes available.

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