

Identification of IgE and IgG binding epitopes on β - and κ -casein in cow's milk allergic patients

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Summary

Background Cow's milk allergy (CMA) affects 2.5% of children aged less than 2 years of age. Although β - and κ -casein are considered among the major allergens responsible for CMA, no data are available on their allergenic epitopes in humans.

Objective The aim of the study was to identify IgE- and IgG-binding epitopes on β - and κ -casein and to determine whether the pattern of epitope recognition is associated with the natural history of CMA.

Methods Overlapping decapeptides representing the entire length of β - and κ -casein, respectively, were synthesized on a cellulose-derivatized membrane. Sera from 15 milk-allergic children, 4–18 years of age, with high levels of specific IgE antibodies to cow's milk were used to identify IgE- and IgG-binding epitopes. In addition, IgE epitopes were screened with pooled or individual sera from younger patients aged less than 3 years and who had low levels of specific serum IgE, who are likely to outgrow CMA.

Results Six major and three minor IgE-binding epitopes, as well as eight major and one minor IgG binding regions, were identified on β -casein. Eight major IgE-binding epitopes, as well as two major and two minor IgG-binding epitopes, were detected on κ -casein. Three of the IgE binding regions on β -casein and six on κ -casein were recognized by the majority of patients in the older age group, but not by the younger patients.

Conclusion Information regarding the immunodominant epitopes in β - and κ -casein may be important for understanding the pathophysiology and natural history of CMA. Differences in epitope recognition may be useful in identifying children who will have persistent milk hypersensitivity.

Keywords: cow's milk allergy, tolerance, epitope, conformational, linear, β -casein, κ -casein, SPOTs membrane

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Introduction

Cow's milk allergy (CMA) is a significant problem in children younger than 3 years of age. Even though cow's milk proteins have been well characterized chemically and physically, there are few data available, especially in humans, regarding their antigenic and allergenic properties.

Caseins are the major allergens responsible for CMA. They account for about 80% of the total protein content in cow's milk. β -casein is the second most abundant protein in cow's milk, constituting 28% of the total milk proteins. The other three casein fractions are α_{s1} -, α_{s2} -, and κ -caseins, and comprise 32%, 10% and 10% of the total protein content, respectively. These proteins have little primary structure homology [1].

In contrast to α_{s1} -casein, there are no data available on allergenic epitopes of β - and κ -caseins in humans. β -casein

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is comprised of 209 amino acids (AA) residues. Although it has little homology to α_{s1} -casein, these two proteins bear some similarities in structure. Both are phosphoproteins, have evenly distributed proline content and lack disulphide bonds. They possess virtually no secondary structure and present diminished tertiary interactions [2,3]. These factors increase the likelihood that the important allergenic epitopes of these proteins are linear rather than conformational.

κ -casein, a lower molecular weight casein, is comprised of 169 amino acids. It differs significantly from other caseins in that it contains bound carbohydrate and possesses disulphide bonds [1,2]. κ -casein has been shown to possess more tertiary structure [4], increasing the likelihood that it bears significant conformational epitopes when compared with α_{s1} -casein and β -casein.

Sensitization to a number of different caseins was observed in the majority of CMA patients [1,5]. It has been suggested that this may be due to a cross-sensitization to common epitopes that exist among caseins, reflecting cross-reactivity. However, the fact that these proteins are encoded by different genes and have very little sequence homology makes it more likely that this is the result of cosensitization. Identification of the immunodominant epitopes of caseins may lead to a better understanding of mechanisms responsible for sensitization to these major milk allergens. Moreover, it is a crucial step in the development of immunotherapy for patients with CMA.

In this study, we used overlapping synthetic peptides to identify major IgE- and IgG-binding epitopes of β - and κ -casein in patients with CMA.

Materials and methods

Patient population

All the patients used in these studies had IgE-mediated CMA. Sera from 15 patients with persistent CMA (11 males, four females) were used to identify IgE- and IgG-binding epitopes. The median age of these patients at the time of collection of sera was 11 years (range 4–18 years), and their levels of milk-specific IgE antibodies ranged from 60 to more than 100 kU_A/L measured by the CAP-System FEIATM (Pharmacia Diagnostics, Uppsala, Sweden). Based on the clinical history of the children in the persistent CMA patient group, eight had atopic dermatitis, five had atopic dermatitis and gastrointestinal symptoms (vomiting and/or abdominal pain), one showed eosinophilic gastroenteritis, and one reacted with anaphylaxis to cow's milk. Ten of the children with presumed persistent CMA were shown by challenge (or accidental ingestion) to still have CMA at the median age of 8.5 years (range 5–14 years). The remaining five patients still avoid cow's milk due to a convincing

history of severe reaction to cow's milk and high levels of milk-specific serum IgE, which have been shown to be highly predictive of positive challenge to cow's milk [6].

Sera obtained from eight patients with CMA (six males, two females) who were less than 3 years of age and had milk-specific IgE levels ranging from 10.8 to 27.6 kU_A/L were also used for identifying IgE binding sites. These children showed low and progressively declining levels of cow's milk-specific IgE, and were therefore considered to be likely to outgrow their CMA. One patient had atopic dermatitis, two had atopic dermatitis and prolonged colic, four had experienced only gastrointestinal symptoms (colic, diarrhoea, protracted vomiting), and one experienced anaphylaxis.

Five non-milk-allergic children (aged 5–11 years) with atopic dermatitis and allergy to other foods (i.e. tomato, shellfish, peanut and nuts) were used as controls.

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

Preparation of SPOTs membrane of overlapping peptides

SPOTsTM membrane (Genosys Biotechnologies, Woodlands, TX, USA), a derivatized cellulose membrane, was used to generate 100 decapeptides for β -casein and 80 for κ -casein, overlapping by eight amino acids and covering the entire sequence of the proteins. Individual peptides were synthesized on the membrane by the 9-fluorenylmethoxycarbonyl (Fmoc) 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 colour reactions during certain steps of the synthesis.

Probing the SPOTs membrane with patient serum

The β -casein membranes were blocked overnight with 1% human serum albumin (HSA) in PBS containing 0.01% Tween 20. For κ -casein, blocking was performed with 1.5% HSA in PBS containing 0.01% Tween 20, and either 2% normal human serum (IgE binding) or 5% rabbit serum (IgG binding). Individual patients' sera were diluted 1 : 50 for IgE binding and 1 : 100 (κ -casein) or 1 : 200 (β -casein) for IgG binding and membranes were incubated with diluted sera for 3 h. Pooled sera from the eight younger patients with low levels of milk-specific IgE were diluted 1 : 2 and incubated overnight for IgE binding to β -casein peptides. Dilutions of sera are based on preliminary experiments in which the intensity of the background was assessed separately for each protein to give a degree of background that would not interfere with the interpretation

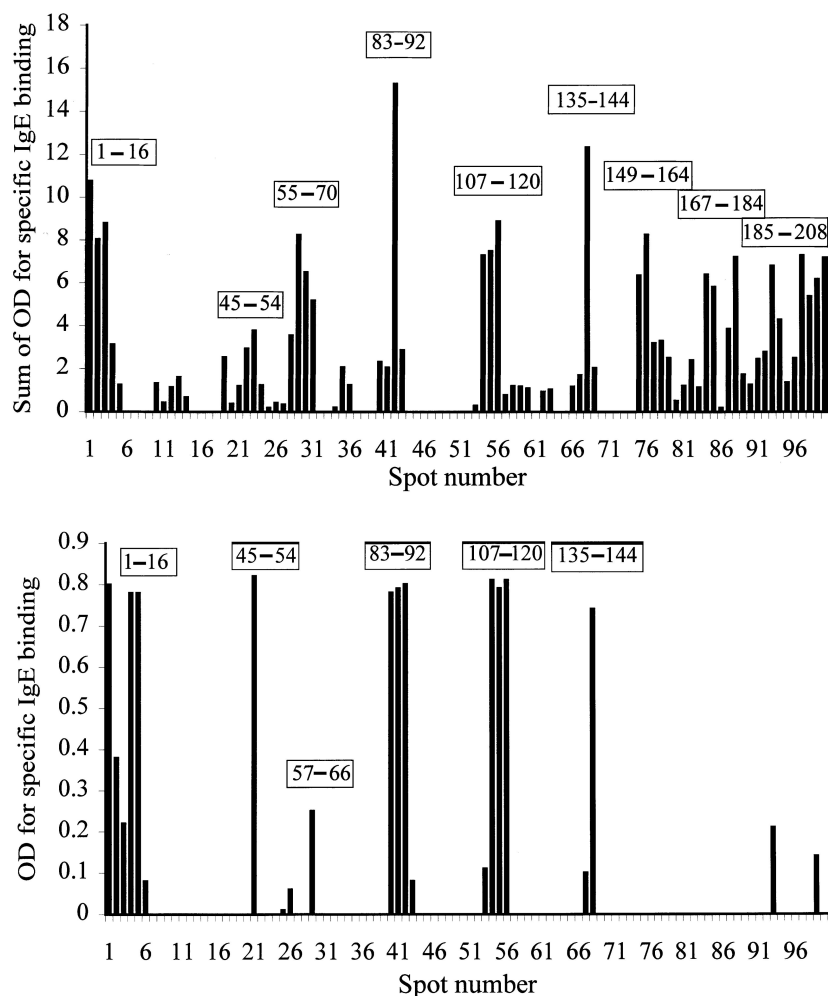


Fig. 1. Cumulative optical density (OD) scores of IgE antibodies for each of the synthetic decapeptides of β -casein. According to the known amino acid (AA) sequence, 100 overlapping decapeptides were synthesized on a cellulose derivatized membrane. Sera from 15 milk-allergic children over the age of 4 years with high levels of specific serum IgE (a) and pooled sera from eight patients with cow's milk allergy who were less than 3 years of age with low levels of milk-specific IgE (b) were used to identify IgE-binding epitopes. Numbers at the tops of the bars represent the individual AA.

of the results. For pooled sera, the sera were diluted less because the levels of IgE antibodies for individual patients were low. IgE and IgG antibody binding was detected by use of an immunoenzymatic method with biotinylated goat antihuman IgE or IgG, and peroxidase streptavidin conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The membranes were developed with a chemiluminescent detection system (Amersham, Arlington Heights, IL, USA).

After developing the X-ray film, optical densities (OD) of each individual peptide spot were 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. The binding of antibodies to each decapeptide was evaluated by calculating individual 'cumulative scores', which represent the sum of the individual ODs from 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 are defined as regions bound by IgE antibodies from at least 50% of the patients. The cut-off level for significant binding was an OD of 0.2 for individual or pooled sera.

Results

IgE-binding epitopes of β -casein

Using individual sera from the 15 older patients with high specific IgE levels, six major and three minor (AA 149–164, AA 167–178 and AA 173–184) IgE binding regions on β -casein were identified (Fig. 1a). Their locations throughout the protein are shown in Fig. 2. Region 1–16 was the most frequently recognized epitope (14/15 patients). The two regions that gave the highest cumulative intensity of IgE binding were regions 83–92 and 135–144 (Fig. 1a), which were recognized by 13/15 and 12/15 of the patients, respectively. The median number of IgE binding

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1          20          40
RELEELNVPGEIVESLSLSSEESITRINKKIEKFQSEEQQQ
TEDELQDKIHFFAQTQSLVYFPGPIPNSLPQNIPPLTOT
          60          80          100          120
PVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT
          140          160          180          200
ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQ
          220          240          260          280
SVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGP
          300          320          340          360
VRGPFPIIV

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Fig. 2. Amino acid sequence of β -casein. Major and minor IgE-binding epitopes are shown in bold italic or italic only, respectively. Major and minor IgG-binding epitopes are underlined with solid or broken lines, respectively.

regions recognized by an individual was six (range 2–9, data not shown).

When labelling the membrane with pooled sera from younger children less than 3 years of age who had low levels of milk-specific IgE, two epitopes recognized by the older patients were not recognized by the younger age group: AA 149–164 and AA 167–184 (Fig. 1b). An additional major epitope at the carboxyl-terminal end (AA 185–208) showed only weak binding with the pooled sera. However, to exclude the possibility that the lack of labelling was the result of lower allergen-specific IgE levels, less diluted sera (dilution 1 : 2) was utilized in the younger children compared with the older children (dilution 1 : 50).

Sera from control patients showed no IgE binding to the peptides (data not shown).

IgG-binding epitopes of β -casein

Using individual sera from the 15 older patients, eight major and one minor (AA 169–184) IgG binding regions on

β -casein were identified (Fig. 3). Their locations throughout the protein are shown in Fig. 2. The two areas with the highest cumulative intensity of IgG binding were regions AA 135–144 and AA 183–208 (Fig. 3). These two areas were recognized by all of the patients. The median number of IgG binding regions recognized by an individual was nine (range 5–9, data not shown). One out of five control patients' IgG bound region AA 25–32 and AA 135–144 but with very low intensity of binding (data not shown).

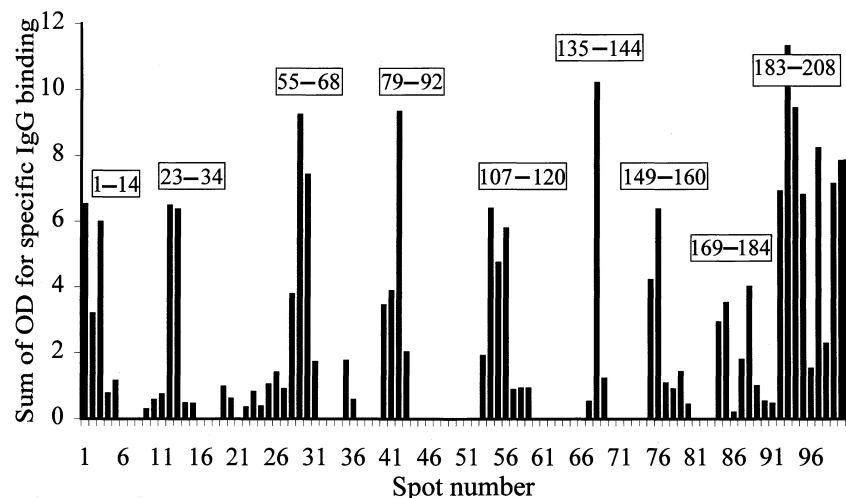
IgE-binding epitopes of κ -casein

Using sera from 15 patients, eight major IgE binding regions on κ -casein were identified (Fig. 4a). Their locations throughout the protein are shown in Fig. 5. The most frequently recognized epitopes were the first three, all of which were recognized by 93% of the patients. These regions also gave the highest cumulative intensity of IgE binding. The median number of major IgE binding regions recognized by an individual was seven (range 2–8). Median cumulative OD score of the whole peptide was 22.8.

When individual patient sera from the eight younger patients were used, only two IgE-binding epitopes were recognized by the majority of the patients (the second and third epitope), with a lower intensity of binding than in the older patients (Fig. 4b). The first, fourth, fifth and seventh epitope were each recognized by only one patient, respectively. The median number of epitopes recognized per individual in the younger patients was two (range 1–4). Median cumulative score of the OD in the younger patients was 2.7. Six linear IgE-binding epitopes were recognized only by the older patients, corresponding to AA 9–26, AA 67–78, AA 95–116, AA 111–126, AA 137–148 and AA 149–166.

Sera from control patients showed no IgE binding to the decapeptides.

Fig. 3. Cumulative optical density (OD) scores for IgG for each of the synthetic decapeptides of β -casein. According to the known amino acid (AA) sequence, 100 overlapping decapeptides were synthesized on a cellulose derivatized membrane. Sera from 15 milk-allergic children over the age of 4 years were used to identify IgG-binding epitopes. Numbers at the tops of the bars represent the actual AA.



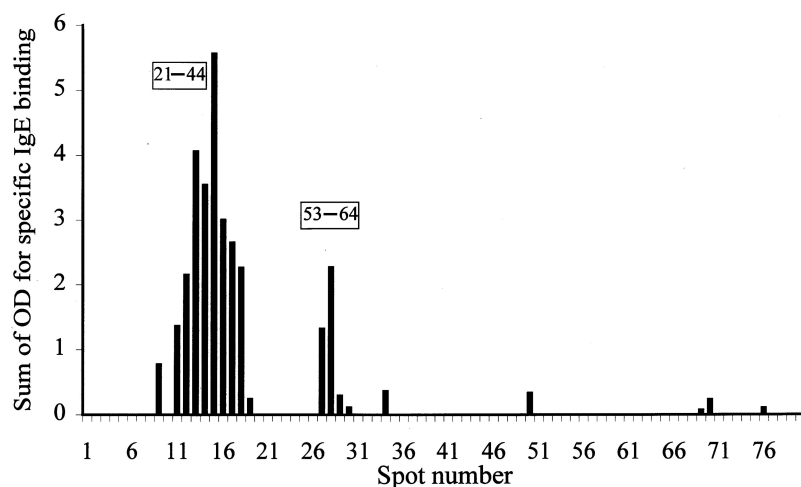
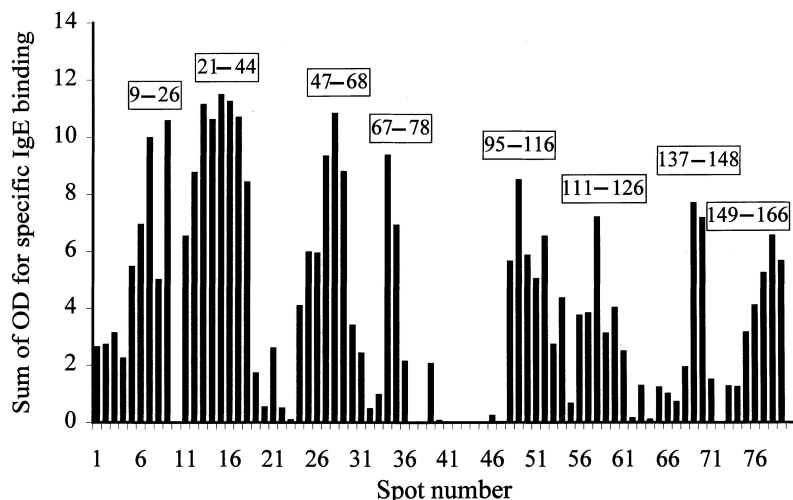


Fig. 4. Cumulative optical density (OD) scores of IgE antibodies for each of the synthetic decapeptides of κ -casein. According to the known amino acid (AA) sequence, 80 overlapping decapeptides were synthesized on a cellulose derivatized membrane. Sera from 15 milk-allergic children over the age of 4 years with high levels of specific serum IgE (a) and from eight patients with cow's milk allergy who were less than 3 years of age with low levels of milk-specific IgE (b), were used to identify IgE-binding epitopes. Numbers at the tops of the bars represent the actual AA.

IgG-binding epitopes of κ -casein

When using sera from 15 patients, two major IgG-binding epitopes were detected on κ -casein (Fig. 6). Two minor IgG-binding epitopes were also recognized (AA 15–24 and AA 37–46) (Fig. 6). The locations of the epitopes throughout the protein are shown in Fig. 5. The median

number of IgG-binding epitopes recognized by an individual was three (range 0–4). The median cumulative OD score of the whole peptide was 4.17. Sera from three control sera failed to bind any epitopes, whereas sera from two control patients showed some binding to all the minor and major epitopes.

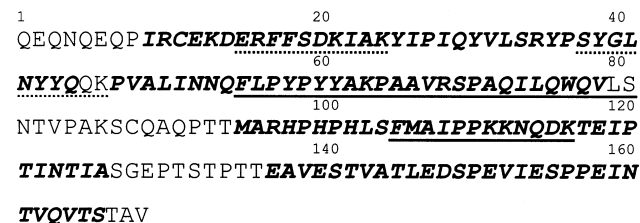


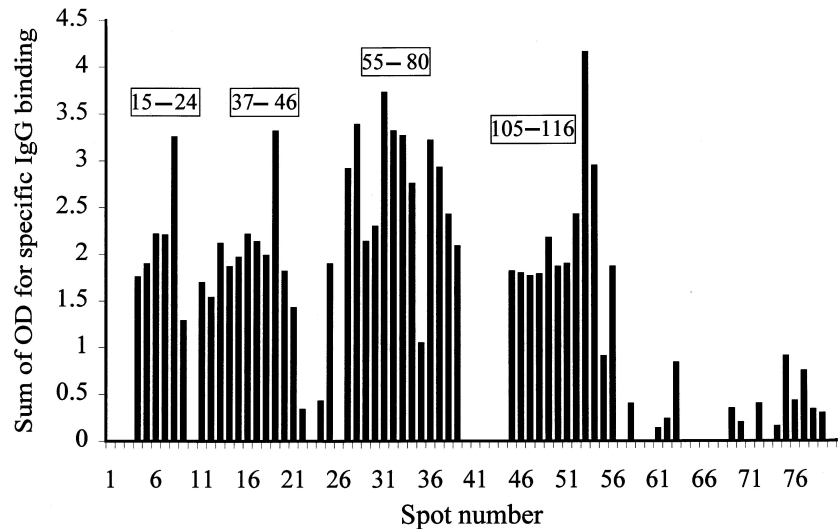
Fig. 5. Amino acid sequence of κ -casein. Major IgE-binding epitopes are shown in bold italic. Major and minor IgG-binding epitopes are underlined with solid or broken lines, respectively.

Discussion

Using overlapping synthetic peptides and sera from CMA patients, we identified immunodominant epitopes on β - and κ -casein, major CM allergens. This is the first report describing allergenic epitopes of bovine β - and κ -casein recognized by allergic individuals.

Previous studies in animal models suggested that multiple epitopes are distributed throughout β -casein. Using sensitized rabbit anti-sera, Otani *et al.* reported the following antigenic regions: AA 1–25, 26–60, 61–93, 94–102, 103–109, 110–144 and 157–185 [7–10]. Using

Fig. 6. Cumulative optical density (OD) scores of IgG binding for each of the synthetic decapeptides of κ -casein. According to the known amino acid (AA) sequence, 80 overlapping decapeptides were synthesized on a cellulose derivatized membrane. Sera from 15 milk-allergic children over the age of 4 years were used to identify IgG-binding epitopes. Numbers at the tops of the bars represent the actual AA.



overlapping peptides and anti- β -casein sera derived from sensitized mice, rabbits and goats, Mizumachi *et al.* identified immunodominant regions for each species and described common antigenic regions among them as regions 1–16, 100–115, 121–136 and 143–158 [11]. In the present study, we identified six major and three minor IgE-binding epitopes, as well as eight major and one minor IgG-binding epitopes. Many regions recognized by human IgE and IgG coincide with the immunodominant regions described in animal studies. Interestingly, we observed that for humans, one major IgE and IgG binding region is located around the carboxyl-terminal end of the protein, while in animals this region was not found to be antigenic.

There is virtually no information regarding IgE- and IgG-binding epitopes of κ -casein, which differs significantly from β -casein in its high degree of conformational structure. A study by Baldo *et al.* [12], using immunoblotting, found that a glycomacropeptide from κ -casein (AA 106–167) and a polypeptide fragment encompassing AA 99–167 were bound by IgE from the majority of milk-allergic children. In our study, we identified four IgE binding regions within the area encompassed by AA 99–167. However, the most frequently recognized IgE binding regions in the present study were located in the *n*-terminal half of the protein, in which we identified another four epitopes that were recognized by most patients.

We also observed differences in epitope recognition between milk-allergic children less than 3 years of age with low levels of specific IgE, who are likely to outgrow their allergies, and the older children with high levels of CM-specific IgE antibodies, who are likely to have persistent CMA. IgE antibodies from the younger group did not recognize two of the epitopes on β -casein and six on κ -casein that were recognized by the older group. This observation supports the hypothesis proposed by Cooke

et al. [13], in which linear epitopes of ovomucoid were associated with the persistence of egg allergy.

Some investigators have suggested that the degree of homology between certain food antigens and human proteins may have some influence on the sensitization and development of allergy to certain food proteins. While caseins account for 80% of the total protein content in cow's milk, they represent only 30–35% of total protein in mature human milk [14,15]. The major casein component in mature human milk is β -casein, which has 66% homology with bovine β -casein. The second most abundant casein in human milk is κ -casein, which has 61% homology with its bovine counterpart. According to our results, several of the major IgE- and IgG-binding epitopes of bovine β -casein have high homology with β -casein in mature human milk. Specifically, the three most frequently recognized IgE epitopes in bovine β -casein (AA 1–16, AA 83–92 and AA 135–144) have 38%, 70% and 80% homology with the corresponding human sequences. The two most frequently recognized IgG epitopes (AA 135–144 and AA 183–208) have 80% and 64% homology with human sequences. For bovine κ -casein, the three most frequently recognized IgE epitopes (AA 9–26, AA 21–44 and AA 47–68) have 53%, 67% and 64% homology, and IgG epitopes (AA 15–24, AA 55–80 and AA 105–116) have 50%, 62% and 92% homology with human κ -casein. Our findings suggest that the high degree of homology may enhance the allergenic and antigenic properties of these peptides. This is in agreement with the study on bovine α -lactalbumin by Maynard *et al.* [16] showing that some of the IgE binding regions were located in the areas with high degrees (81% and 87%) of homology to human α -lactalbumin.

The information on IgE- and IgG-binding epitopes of β - and κ -caseins identified in this study represents further

steps in the delineation of the allergenic and antigenic structures of cow's milk proteins. The information provided may be useful in the development of more specific diagnostic methods of CMA and eventually lead to safe, effective therapeutic approaches for patients with CMA.

Acknowledgements

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