

Immunochemical and molecular characterization of milk allergens

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Cow's milk allergy (CMA) is usually developed in early infancy. Children are said to outgrow their milk intolerance reactions. However, CMA also occasionally occurs in adults (1, 2).

Cow's milk allergens

Cow's milk contains about 30-35 g of proteins (CMP) per l. The action of an enzyme, chymosin (rennin), or the acidification of the milk to pH 4.6 enables two fractions to be obtained: lactoserum (whey), which contains about 20% of the proteins, and coagulum (curd), which contains the caseins accounting for about 80% of the milk proteins. Whey contains essentially globular proteins. The major ones, i.e., β -lactoglobulin and α -lactalbumin, are directly synthesized in the mammary gland, while others, such as bovine serum albumin, come from the blood. The casein fraction comprises four proteins coded by different genes carried on the same chromosome: α_{s1} -, α_{s2} -, β -, and κ -caseins. They form ordered aggregates: micelles in which their proportion is relatively constant at about 37%, 37%, 13%, and 13%, respectively, of the protein content of the whole-casein fraction. They have little primary structure homology. However, the four caseins display common features; e.g., a dipolar-type structure with amphipathic properties. They are phosphorylated proteins, with a loose tertiary, highly hydrated structure, very susceptible to all proteinases and exopeptidases, and are therefore considered poorly immunogenic (3).

It has classically been accepted that, in CMA, the major allergen is β -lactoglobulin. However, this view has now been completely undermined, and sensitivities to the various CMP have been proved

to be widely distributed (4, 5). A study on 92 sera from allergic patients showed that most of the patients were sensitized to several proteins; e.g., β -lactoglobulin (b-Lg), whole-casein fraction (cas), α -lactalbumin (a-Lac), bovine serum albumin (BSA), lactoferrin (Lf), and immunoglobulins (Ig). A great variability was observed in the affinity, specificity, and magnitude of the IgE response. The main proteins by weight, such as cas and b-Lg but also a-Lac, appeared to be major allergens since 66%, 68%, and 58%, respectively, of patients were specifically sensitized to these proteins. However, proteins present in very low quantities, such as BSA, Igs, and especially Lf, also appeared to be of great importance, since 50%, 36%, and 45% of patients were respectively sensitized to these proteins, which sometimes were the only ones recognized by some patients in whom they induced clinical manifestations of food allergy (6).

Identification of allergenic determinants on CMP molecules

The primary structure and part of the secondary and tertiary structure of CMP are known. However, caseins cannot be crystallized, and their tertiary structure is approached by molecular modeling.

β -Lactoglobulin (b-Lg)

This occurs naturally in the form of a 36-kDa dimer. It has no homologous counterpart in human milk. Each subunit corresponds to a 162-residue polypeptide. The molecule possesses two disulfide bridges and one free cysteine. This structure is

responsible for the relative resistance of b-Lg to acid hydrolysis, as well as to proteases, which allows some of the protein to remain intact after digestion and increases the probability that intact b-Lg as well as digested fragments will be absorbed as antigens. These properties led researchers to consider b-Lg the major milk allergen. There are two main isoforms of b-Lg; i.e., genetic variants A and B, which differ only by two point mutations on residues 64 and 118, aspartic acid and valine in b-Lg A, and glycine and alanine in b-Lg B, respectively (3).

The tertiary structure of b-Lg is known and the spatial conformation has recently been determined. It belongs to the lipocalin family and is considered a retinol-binding protein. Lipocalins share well-conserved sequence homologies in their N-terminus moiety, where tryptophan at position 19 is always present. Crystallography studies revealed very similar folding, called β barrel structure, with the same arrangements of eight (or 10) antiparallel β sheets, in spite of the mutations that have occurred during evolution (7). This kind of molecule has a high allergenic potential, and several allergens of animal origin belong to this family such as the major mouse (and rat) urinary proteins (mMUP), the major horse allergen Equ c 1, and the major cockroach allergen Bla g 4 (8).

Although the structure of the two variants A and B is very similar, the intensity and duration of the IgE response in the rat sensitized by peritoneal injection are greater in the case of b-Lg B. On the other hand, in guinea pigs immunized orally, the IgG1 or IgG2 response is stronger against b-Lg A than against the variant B (9).

Few studies on the relationship between structure and allergenicity and on epitope analysis have been performed with human sera from patients with CMA. Cleavage of the intrachain disulfide bonds within the b-Lg molecule and consequently the loss of the conformation of the molecule had little if any effect on its immunoreactivity, suggesting that linear epitopes are implicated (10, 11). Selo et al. have observed that sensitization involved many epitopes that are widely spread all along the b-Lg molecule. Some of them were identified as short linear sequences, while other immunoreactive structures corresponded to quite large fragments that might encompass conformational epitopes or parts of epitopes (11). The IgE-binding capacity of fragments 124–134 and 149–162 have been demonstrated with synthetic peptides (12, 13); moreover, the major role of histidine 161 has been highlighted, the substitution of which causes the fragment to lose most of its antigenicity (13). A linear epitope corresponding to sequence 97–108 was recognized

by IgE from a high proportion of patients with CMA (14).

It is generally accepted that the hydrolysis of milk proteins considerably reduces their allergenicity. However, Haddad et al. (15) have shown that specific IgE from 10 patients with CMA recognized enzymatic digestion products of b-Lg by pepsin or pepsin + trypsin (10 patients out of 10) and that the recognition of peptides was even better than that of the intact molecule in four patients out of 10. They concluded that the digestive processes unmask new allergenic epitopes. Selo et al. confirmed that cleavage may allow the presentation of determinants which were not accessible to the antibodies on the whole native protein (11).

α -Lactalbumin (a-Lac)

α -Lactalbumin (a-Lac) is a monomeric globular protein of 123 amino-acid residues with molecular weight of 14.4 kDa and four disulfide bridges (3). The peptide comprising loop 60–80, which possesses a disulfide bridge, is considered an antigenic region on the animal model. However, it was not identified as a major epitope for human IgE in a study on sera from 19 patients with CMA, which used purified tryptic digest fragments of a-Lac (16). IgE binding to native a-Lac and to large peptides confirmed the importance of conformational epitope(s). However, in some sera, reduced peptide, e.g., 59–94, exhibited a similar or a higher IgE-binding capacity than the native corresponding fragment, suggesting the existence of sequential epitope(s) exposed through protein denaturation. Moreover, IgE-binding sequences were also located in hydrophobic regions of the a-Lac molecule, where antigenicity is very unlikely to be predicted, and/or within parts of the molecule having a very high sequence homology with human a-Lac.

Caseins

A striking common characteristic in α - and β -caseins is the homology in the acidic peptide sequence containing the cluster of phosphoserine residues: 13–21 in β -casein and 62–70 in α_{s1} -casein. The phosphate groups seem to be localized at the β turns, and the folding of the molecule brings them into proximity with each other to form a very hydrophilic domain. Similar sequences, e.g., 7–12 and 55–60, appear twice in α_{s2} -casein variant A.

The specificity of the IgE response to the different purified caseins has been analyzed on 58 sera from patients allergic to the whole-casein fraction (17). Multisensitizations were observed which probably corresponded to a cosensitization

to the different casein components after the disruption of the casein micelles. However, cross-sensitization mechanisms also occurred through common or closely related epitopes. As already noticed, the region containing the major site of phosphorylation is conserved in bovine α_{s1} -, α_{s2} -, and β -caseins, as well as in the caseins of other species. Hence, this sequence may play an important part in cross-reactivity, especially since it has already been described as being immunoreactive, and resistant to digestive degradation (18). Using overlapping synthetic decapeptides, Spuerger et al. (19) have described three sequences, e.g., peptides 19–30, 86–103, and 141–150, recognized by sera of all of the 15 patients with CMA, the most immunoreactive one being peptide 86–103. These three major epitopes are located in hydrophobic regions of the molecule, where they are not accessible to antibodies unless the casein is denaturated or degraded, e.g., during digestion.

It must be stressed that casein "allergenic epitopes" may be present in whey! Indeed, hydrolysis of a fraction of the casein, e.g., β -casein, naturally occurs due to endogenous enzymes such as plasmin normally present in milk. Splitting of the molecule gives rise to γ -caseins, and to smaller fragments called proteoses-peptones corresponding to the N-terminal part of the β -casein molecule. These peptides are soluble and remain in the lactoserum. Similarly, the limited proteolysis due to the action of chymosin during clotting of milk splits κ -casein into two peptides: hydrophobic para κ -casein (1–105) and highly polar caseino-macro-peptide (106–169), which is soluble and remains in the whey. Some proteoses-peptones are still allergenic, as well as caseino-macro-peptide, which explains why reactions may be observed after ingestion of whey protein, hydrolysates in babies having a RAST[®] positive to casein but negative to whey proteins.

Conclusions

Due to the great variability of the human IgE response, no single allergen or particular structure can account for a major part of milk allergenicity. The main characteristic that can be underlined is the multiplicity and diversity of allergens and, for each allergen, of the molecular immunoreactive structures (e.g., "epitopes") that are involved in CMA. These epitopes appear to be numerous and widely spread along the protein molecule. They are often located in hydrophobic parts of the molecule, inaccessible to IgE antibodies in the native conformation of the protein, but become bioavailable after digestive processes. Three-dimensional structure is an important feature in CMP allergenicity.

but besides the conformational epitopes, IgE-binding studies also showed the presence of linear epitopes. Peptides as short as about 12–14 amino-acid residues have been characterized to account for a significant part of the allergenicity of the whole molecule in some patients. This observation justifies the need for caution before using peptides for hyposensitization or proposing any milk protein hydrolysates in the diet for highly allergenic children.

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