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An update on allergens

Cow's milk allergens

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Cow's milk allergy (CMA) is mainly an IgE-mediated hypersensitivity reaction, although other immune mechanisms or clinical (delayed or late) reactions have also been identified (1). In an extensive review, Host (2) noted that estimates of the total incidence of reactions to cow's milk vary greatly with differences in study design, criteria for diagnosis, and geography. Moreover, figures actually most often refer to adverse reactions which may be caused both by type I immunologic mechanisms (CMA, strictly speaking) and by cow's milk intolerance. Incidence thus varies from 0.1 to 7.5% (3, 4). Cow's milk allergy/intolerance was diagnosed in 1.9–2.8% of general populations of infants in the first 2 years of life in different countries of northern Europe (5, 6), but its incidence fell to approx. 0.3% in children older than 3 years (7). Cow's milk allergy/intolerance is usually developed in early infancy, and children are said to outgrow their milk intolerance reactions. However, CMA also occasionally occurs in adults. Björnsson et al. (8) found a prevalence of 1% of CMA in adults in Sweden, and, interestingly, in a retrospective study on 34 adults allergic to milk and dairy products who were diagnosed in Switzerland between 1981 and 1991, women represented 91.2% of the study group (9).

Cow's milk protein composition and IgE repertoire

Cow's milk protein (CMP) composition and some characteristics of the major proteins are shown in Table 1, which is adapted from Jost (10).

Cow's milk contains about 30–35 g of protein per litre. 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, which have little primary structure homology. Their functional properties also differ, since three of them, α_{S1}-, α_{S2}-, and β-casein, appear to be calcium-sensitive, while κ-casein is not. However, the four caseins display common features that are quite unusual, differing greatly from other typical milk proteins. They are phosphorylated proteins, with a loose tertiary, highly hydrated structure. Casein (e.g., whole casein fraction) is often considered poorly immunogenic because of this flexible, non-compact structure. Moreover, after ingestion, most of this structure and the presentation of the potential antigenic and/or allergenic fractions are assumed to be modified by acidic gastric conditions, the action of digestive enzymes, and uptake through the intestinal mucosa. Caseins are not significantly affected by severe heat treatments but are indeed very susceptible to all proteinases and exopeptidases. Each casein represents a well-defined chemical compound, but the different

Table 1. Concentration and properties of main allergenic proteins in cow's milk (adapted from Jost [10])

Proteins (30–35 g/l)		Concentration (g/l)	kDa	No. amino-acid residues per molecule	No. S–S bridges per molecule	Secondary structures (% of total chain)		pI
						α-helix	β-sheet	
Whey (20%)	b-Lg (10%)	3–4	18.3	162	2 +1 free SH	+++	15	5.3
	a-Lac (5%)	1–1.5	14.2	123	4	++	25	4.8
	Igs (3%)	0.6–1.0	150					
	BSA (1%)	0.1–0.4	66.3	582	17 +1 free SH	+++	50	4.9–5.1
	Lf (traces)	0.09	80	703	16			8.7
Casein (80%)	α_{S1}-Casein (32%)	12–15	23.6	199		+	4–15	4.9–5
	α_{S2}-Casein (10%)	3–4	25.2	207	1			5.2–5.4
	β-Casein (28%)	9–11	24.0	209		+	1–10	5.1–5.4
	κ-Casein (10%)	3–4	19.0	169	1	++	14	5.4–5.6

caseins associate with each other in solution. They cross-link to form complexes and then ordered aggregates: micelles, in suspension in the aqueous phase or lactoserum. The proportion by weight of the α_{S1}-, β-, α_{S2}-, and κ-caseins in the micelles is relatively constant: approx. 37, 37, 13, and 13% of the protein content of the whole-casein fraction (11). On the other hand, their distribution is not uniform within these micelles. The different caseins are assembled and organized by intermolecular interactions between the colloidal calcium phosphate and the phosphoserine groups of the α_{S1}-, α_{S2}-, and β-caseins. They form globular complexes which comprise a central hydrophobic part and a peripheral hydrophilic layer where the C-terminal polar fragment of the κ-casein molecule (e.g., caseinomacropeptide) is exposed. Polar domains of the other caseins (α_{S1}-, α_{S2}-, and β-) and, notably, major sites of phosphorylation containing phosphoserine residues are also presented at the periphery of the micelles (12).

Several forms of caseins have been described which derived from post-translational modifications such as phosphorylation, glycosylation, or partial hydrolysis (13).

Finally, the heterogeneity of the milk proteins is complicated by their genetic polymorphism, resulting in several variants of each protein, which can be separated and identified by electrophoresis. These variants are characterized by point substitutions of amino acids or by deletions of peptide fragments of varying size in the primary sequence (14).

Classically, it has been accepted that the major allergen in CMA is β-lactoglobulin. This protein is normally absent from human breast milk. Its resistance to acidic pH values and to proteolytic enzyme cleavages leaves its structure relatively unchanged during digestion, allowing a possible passage into the blood in an intact form (see below).

However, this view has now been completely undermined, and sensitivities to the various CMP have been proved to be widely distributed (15, 16).

The IgE repertoire is the whole set of constituents of milk, or the subfractions thereof, that IgE from patients with CMA are specifically able to recognize and to bind. Various electrophoretic techniques associated with immunoblotting have been used to analyze this repertoire. Gjesing et al. identified specific IgE against four major whey proteins and against three caseins in the serum of 21 cow's milk-allergic patients by cross-radioimmunoelectrophoresis (17). Two-dimensional immunoelectrophoresis has been used as a global approach to determine allergenic components in skimmed milk. The spots obtained upon electrophoresis can be identified through knowledge of isoelectric points and of the molecular weights of the different milk proteins. Several constituents of milk have thus been revealed as allergens; e.g., they were recognized by specific IgE from patients (18).

RAST or derived ELISA tests or skin prick tests using purified proteins, sometimes confirmed by oral challenge, have also permitted researchers to outline the incidences of sensitization to the main CMP (19, 20). Specific and quantitative ELISA tests for determination of specific IgE to highly purified isoforms of milk proteins, e.g., β-lactoglobulin (Bos d 5) variant B (b-Lg), whole-casein fraction (Bos d 8) (cas), α-lactalbumin (Bos d 4) (a-Lac), bovine serum albumin (Bos d 6) (BSA), lactoferrin (Lf), immunoglobulin (Bos d 7) (Igs), have revealed great variability in the affinity, specificity, and magnitude of the IgE response (21). A study on 92 sera from allergic patients (22, 23) showed that most of the patients were sensitized to several proteins. Only 27% were monosensitized. Most of them were monosensitized

to either cas, b-Lg, or BSA, but some were monosensitized to a-Lac, and even to Igs, or to Lf. In terms of multisensitivities, 15, 20, 22, and 16% of the 92 allergic patients were sensitized to two, three, four, and five allergens, respectively. Most of the possible combinations of allergens were observed.

The main proteins by weight, e.g., cas and b-Lg but also a-Lac, appeared to be major allergens, since 66, 68, and 58% of patients were specifically sensitized to these proteins, sometimes displaying high specific IgE titers. These figures are in good agreement with other studies that particularly stressed that the incidence of casein as allergen has become very important in terms both of frequence and intensity of IgE response (9, 24). However, proteins present in very low quantities, such as BSA, Igs, and especially lactoferrin, also appeared to be of great importance, since 50%, 36%, and 45% of patients, respectively, were sensitized to these proteins, which sometimes were the only ones recognized by the IgE of certain patients, in whom they induce the clinical manifestations of food allergy. In such cases, confirmation of the CMA and identification of the responsible allergen require very specific and sensitive immunochemical tests for detection of specific IgE to these minor proteins.

Anticasein IgE were shown to be present at the same time with anti-a-Lac IgE in 87% of sera. Sensitizations to b-Lg and to a-Lac were also closely related. In contrast, sensitization to BSA appeared to be completely independent, 50% of the patients being sensitized to BSA regardless of their sensitization to other milk allergens (22).

Structure of milk proteins and identification of allergenic determinants on the molecules

The primary structure and part of the secondary and tertiary structures of CMP are known (Fig. 1). However, caseins cannot be crystallized, and their tertiary structure is approached by molecular modeling.

Epitopic analysis of CMP has given rise to numerous studies carried out with direct or competitive inhibition ELISA tests with antibodies (IgE or IgG) from animals experimentally sensitized. The doses and the modes of administration differ from one study to another and, in particular, rarely correspond to the conditions of a digestive sensitization in atopic patients. This imperfect tool gave very variable results which may even contradict the few studies performed on allergic human sera.

Whey proteins

The major allergens of lactoserum are b-Lg and a-Lac.

β-Lactoglobulin (b-Lg). b-Lg 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 (25). This structure is responsible for the main physicochemical properties already mentioned and also for interaction with casein during heat treatments (26). The relative resistance of b-Lg to acid hydrolysis, as well as to proteases, allows some of the protein to remain intact after digestion. This increases the probability that intact b-Lg, as well as digested fragments, will be absorbed as antigens. Indeed, b-Lg, from ingested cow's milk, can be detected in human milk and may cause colic in breast-fed infants (27).

There are two main isoforms of b-Lg, i.e., the genetic variants A and B, which differ only in two point mutations on residues 64 and 118; aspartic acid and valine in b-Lg A, and glycine and alanine in b-Lg B (28).

The tertiary structure of b-Lg is known. It belongs to the lipocalin family and is considered a retinol-binding protein (29). The spatial conformation has recently been determined (30). Like other related proteins, such as fatty acid-binding proteins, lipocalins bind and carry hydrophobic ligands, such as retinol. They share well-conserved sequence homologies in their N-terminus moiety, where tryptophan at position 19 is always present. The tertiary structure is very similar and has a very high affinity for hydrophobic ligands. 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 (31). This kind of molecule has a high allergenic potential, and several allergens of animal origin belong to this family. The major mouse (and rat) urinary proteins (mMUP) are very potent allergens present in the urine but also the saliva of mice and rats. They are α_2-microglobulins of similar structure. The major horse allergen, Equ c 1, has also been characterized as a highly homologous α_2-microglobulin which binds retinol. A major allergen from cockroach, Bla g 4, also presents a very similar structure and conformation (32).

Although the structure of the A and B variants 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 to b-Lg A than to the variant B (33).

The immunoreactivity of native and denaturated b-Lg has been compared with the use of specific antibodies from mice after intraperitoneal immunization (34), rat anti-b-Lg IgE (35), and human specific IgE from patients with CMA (36). Cleavage of the intrachain disulfide bonds within the b-Lg molecule and consequently the loss of the conformation of the molecule had little or no effect on its immunoreactivity in the three models, suggesting that linear epitopes are mainly implicated. This is in contrast with the results obtained with specific rabbit anti-b-Lg IgG, which recognized only the native molecule and thus antigenic determinants comprising conformational sites (35).

Few epitope analyses have been done with human sera from patients with CMA. These studies determined only the specificity of either one serum against various peptides (37) or of a limited number of sera against a single epitope (38, 39).

The hydrophilicity profile of b-Lg suggests the antigenicity of sequence 124–134. This peptide possesses one of the rare common sequences with α-lactalbumin and may be responsible for the IgE cross-reactivities sometimes observed between a-Lac and b-Lg. Using the serum of one patient allergic to b-Lg, Adams et al. showed that the IgE-binding capacity of the corresponding synthetic peptide was 60% of the binding of the whole b-Lg (38). Tokita had already reported a similar sequence, e.g., 125–135, as well as fragment 41–61, as an antigenic determinant (40).

For their part, Mizumachi et al. identified six specific antibody-binding sites on the b-Lg molecule. The use of synthetic peptides which mimic the C-terminal region allowed them to show the antigenicity of fragment 149–162 and to highlight in this peptide the major role of histidine 161, the substitution of which causes the fragment to lose most of its antigenicity (41). Incidentally, the peptides 42–56 and 72–86, identified as B-cell antigenic determinants, were also recognized by the same group as T-cell determinants in the same relevant mice strains (42).

Takahashi et al. described four epitopes, including fragment 149–162. As already mentioned, the four fragments kept their immunoreactivity in the reduced and *S*-carboxymethylated b-Lg molecule; e.g., after loss of its spatial configuration. They also showed that numerous isolated tryptic peptides kept their antigenicity (34). In this, they were in conflict with the earlier results of Huang et al. (35), but confirmed some of the very many observations of Otani et al. (37, 43) and Otani & Hosono (44). These authors had identified numerous antigenic regions, which differed partly from the previous ones and also varied between the different studies, depending on the animal/human model used. However, this team did find the sequence 146–162 observed by several authors and noted also that four regions kept their reactivity in the denatured b-Lg.

Using overlapping peptides, Ball et al. have described a linear epitope which was recognized by IgE in a high proportion of sera from allergic patients. They synthesized the corresponding dodecapeptide, allowing them to localize the IgE-binding region of the molecule on sequence 97–108 (39).

Selo et al. have recently studied the IgE-binding capacity of four fragments obtained by CNBr cleavage of b-Lg on the sera of 19 allergic patients (36). CNBr splits the protein molecule at the methionine residues, giving rise to four fragments: 8–24, 108–145, 25–107, and 146–162. These fragments were isolated and purified and were shown to account for a variable but significant part and sometimes even all the immunoreactivity of the whole native (or denaturated) molecule, depending upon the patient and the peptide. Most sera recognized several peptides, and the human IgE response appeared to be very variable and heterogeneous.

Sensitization thus involved many epitopes that were 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.

These results on inhibition of IgE binding to b-Lg by isolated fragments are mostly obtained in the presence of large quantities of peptides, and it is generally accepted that the hydrolysis of milk proteins considerably reduces their allergenicity (10, 45). For this reason, it is interesting to recall the work of Haddad et al., who have shown that specific IgE from 10 patients with CMA recognized enzymatic digestion products of b-Lg by pepsin or pepsin plus 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 (46). They concluded that the digestive processes unmask new allergenic epitopes. Selo et al. confirmed that CNBr hydrolysis of b-Lg may affect its immunoreactivity to specific IgE from some allergic patients. However, for 50% of the patients, CNBr cleavage had low or even no effect, suggesting that a large part or all of the epitopes was included in the fragments. Moreover, in 10% of the sera, chemical cleavage enhanced protein immunoreactivity, suggesting that cleavage allowed the

presentation of determinants which were not accessible to the antibodies on the whole native protein.

α-Lactalbumin (a-Lac). α-Lactalbumin (a-Lac) is a monomeric globular protein of 123 amino-acid residues with a molecular weight of 14.4 kDa and four disulfide bridges (47). The complete amino-acid sequence of bovine a-Lac shows extensive homology with human a-Lac, since 74% of the residues are identical and another 6% chemically similar (48). However, despite this great similarity, bovine a-Lac has been identified as a major cow's milk allergen. It possesses a high-affinity binding site for calcium, and this bond stabilizes its secondary structure. It is a regulatory component of the enzymatic system of galactosyl transferase that is responsible for the synthesis of lactose (11).

On the basis of their amino-acid sequences and three-dimensional structures, a-Lac and hen's egg white lysozyme are closely homologous, having evolved by divergence from a common ancestral gene (49, 50).

A theoretic analysis based on profiles of the hydrophilicity of the molecule, calculated according to the algorithm of Hopp & Woods (51), shows two peaks at residues 5 (lysine) and 15 (leucine). This has suggested that the major antigenic site is on peptide 5–18 (38). This is one of the regions where the difference in sequence is greatest between bovine and human a-Lac. This peptide, which corresponds to the homologous sequence 124–134 on the b-Lg molecule, has been synthesized and has indeed shown a large capacity for binding with specific IgE antibodies from two human sera (38).

Using rabbit antisera, Hopp & Woods have studied the antigenic activity of fragments derived from peptic digestion of a-Lac (52). They identified a highly antigenic region accounting for 20–25% of whole a-Lac antigenicity at the peptide comprising loop 60–80, which possesses a disulfide bridge and is itself attached by another disulfide bridge to peptide 91–96. The same structure is found in sheep a-Lac. Other peptic fragments were also found to be antigenic. Maynard et al. have investigated the human IgE-binding capacity of tryptic digest fragments of a-Lac on the sera of 19 allergic patients (53). Ten and five highly purified peptides, respectively, were obtained after tryptic cleavage of either native or disulfide bonds reduced (e.g., denatured) a-Lac. Eleven sera recognized only the whole protein, while eight had IgE able to bind isolated tryptic fragments. The "peptide loop" considered an antigenic region on animal models was not identified as a major epitope for human IgE. Sequence 17–58 was most strongly and frequently recognized, but clear evidence of binding to smaller peptides, e.g., 6–10:S–S:115–123 or 109–123, was sought. The authors noted that IgE

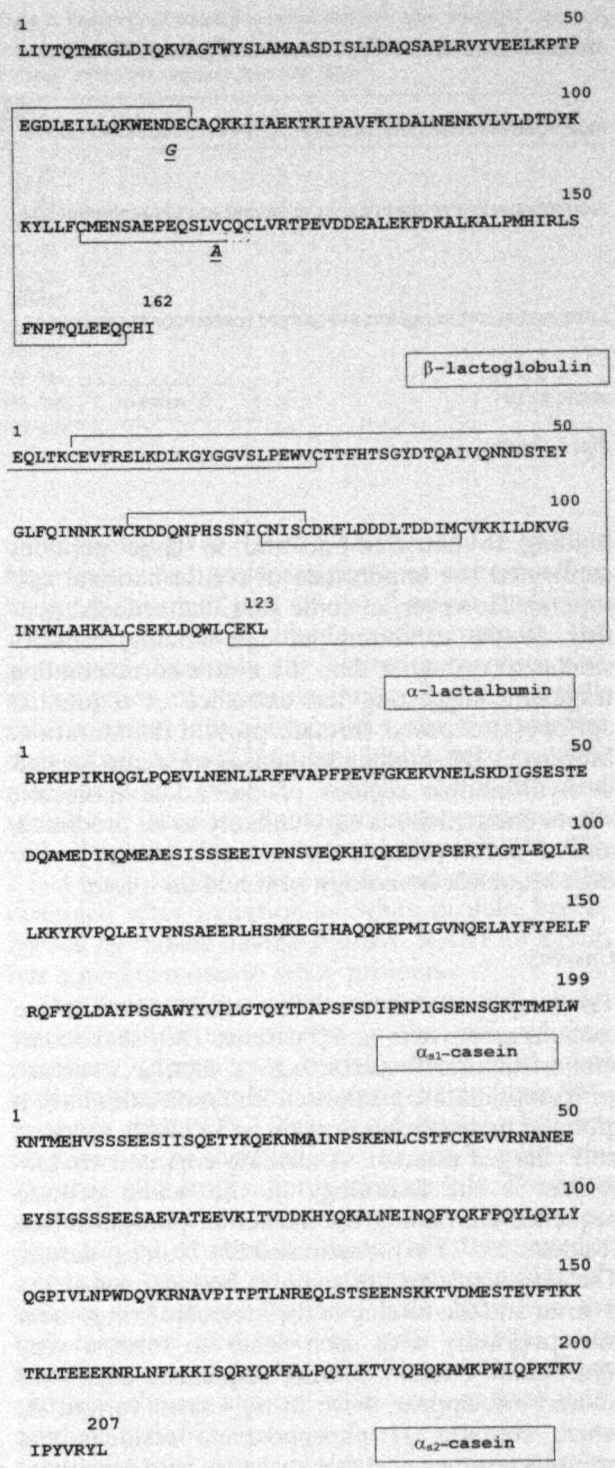

Fig. 1. Amino-acid sequences and location of disulfide bridges of major cow's milk allergens: β-lactoglobulin: variant A and *variant B* (from Mckenzie et al. [25]); α-lactalbumin (from Brew et al. [47]); α_{S1}-casein, (from Mercier et al. [69]); α_{S2}-casein (from Brignon et al. [70]); and β-casein (from Ribadeau-Dumas et al. [71]).

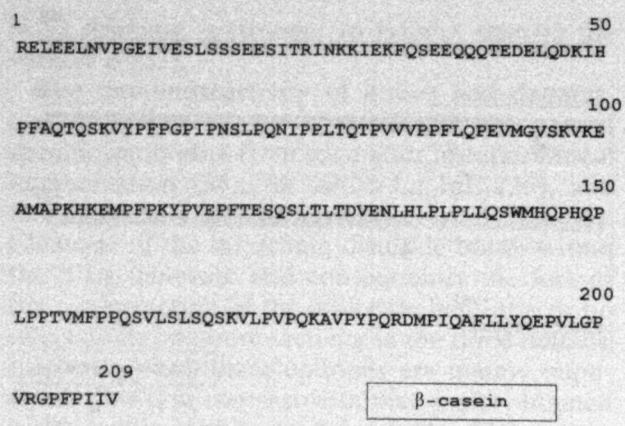

Fig. 1. cont.

binding to native a-Lac and to large peptides confirmed the importance of conformational epitope(s). However, in some sera, the reduced peptide 59–94 exhibited an IgE-binding capacity similar to or higher 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

The major allergens of the casein fraction are calcium-sensitive α- and β-caseins. They share common structural features; e.g., a dipolar structure with amphipathic properties. The molecules have a globular hydrophobic domain and a highly solvated and charged domain. A striking common characteristic 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, which contains 11 phosphoserine residues and presents a rather unstable structure with sensitivity to pH. One of the genetic variants of α_{S2}-casein (e.g., variant D) results from a deletion of the amino-acid sequence 51–59. This deletion removes a cluster of three phosphoserine residues, so that the structural characteristics and probably the allergenic potential may be different in the two α_{S2}-casein variants. β-Casein possesses five main genetic variants with different levels of phosphorylation. The α- and β-casein molecules contain various numbers of proline and hydrophobic residues. They have no rigid tertiary structure but do have a "random coil" conformation which is stabilized by hydrophobic interactions; thus, they are considered poorly immunogenic (11, 13, 14).

Recently, Bernard et al. have studied the specificity of the IgE response to the different purified caseins, on 58 sera from patients allergic to the whole-casein fraction (54). Only one case of monospecificity (to κ-casein) was observed, while specific IgE against each of the four caseins could be detected in most of the sera.

The intensity of the IgE responses greatly varied between the sera and between the caseins but seemed closely related to the proportion of the four caseins in milk. Interestingly, β-casein, which is abundant in human milk and exhibits substantial homology with its human counterpart, induced a high IgE response. These results probably corresponded to a cosensitization to the different casein components after the disruption of the casein micelles. However, polysensitization also appeared to be due to cross-sensitization mechanisms, since, in some sera, the sum of the IgE titers against the different caseins largely exceeded the IgE titer to the whole casein, suggesting the presence of IgE antibodies cross-reacting with different caseins through common or closely related epitopes. As already noted, 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 (55) and resistant to digestive degradation (56).

α-Caseins. The numerous publications of Otani et al. on peptides resulting from the hydrolysis of α_{S1}-casein by cyanogen bromide and trypsin have highlighted the role of the phosphorylated regions in antigenicity, describing six allergenic and/or antigenic fragments, of which the main one is the large peptide 61–123 (55, 57, 58). Ametani et al. only confirmed peptide 133–151, already described by Otani et al., but found no immunoreactivity on tryptic or chymotryptic peptides containing the sequences of phosphoserine residues (59). Kaminogawa's group also identified limited determinants as being responsible for the immunoreactivity of the α_{S1}-casein. They highlighted the role of peptide 91–110, both as B-cell antigenic determinant and T-cell-activating determinant in mice (60, 61). Spuergin et al. used overlapping synthetic decapeptides in immunoblot and direct or competitive ELISA techniques to study the regions of the αs_1-casein molecule immunoreactive to IgE and IgG from allergic human subjects (62). No major difference

was noticed between IgE and IgG specificity. Three sequences, e.g., peptides 19–30, 86–103, and 141–150, were 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 denatured or degraded. In whole casein, the hydrophobic parts of the molecules may be involved in the interactions stabilizing the complex or may be hidden in the core of the complex. During digestion, physicochemical modifications induced by the different digestive media and by enzymatic cleavages might lead to exposure to the immune system of some zones initially inaccessible, and IgE subpopulations could then be produced against these sequences.

Some other peptides were also recognized, but only weakly and only by a few patients. In contrast, these minor epitopes were located in very hydrophilic parts of the molecule, exposed at its surface, particularly on the negatively charged peptide 58–73, which contains four phosphorylation sites. However, this low reactivity could be explained by the fact that serine residues were not phosphorylated in the synthetic peptides.

β-*Casein.* The study of the antigenic and allergenic structures has given rise to various papers by Otani et al. (37, 63, 64). The results were very variable, depending on the experimental conditions. They are presented in Table 2. It can be noticed that all the antigenicity of the native molecule of β-casein is found in the sum of the antigenicities of fragments resulting from its hydrolysis by cyanogen bromide and/or trypsin. This additivity led to the conclusion that at least six antigenic sites exist on the β-casein molecule and that the epitopes are sequential. The authors noticed that sequence –K–X–K–E– is found in peptides 94–102 and 103–109. Similar sequences containing one acidic and two basic amino-acid residues are also present in antigenic regions of the lysozyme molecule. They also observed that glutamic acid residue 121 or 131 seems to be necessary to maintain the antigenicity of the sequence 110–144 and stressed that sequence 14–21, which comprises four phosphorylated serine residues, is involved in the antigenicity of fragment 1–25. On the other hand, the peptides were not all recognized in the same manner; this depended on the isotypes of antibodies used for their detection.

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: the hydrophobic para-κ-casein (1–105) and the highly polar caseinomacropeptide, 106–169, which is soluble and remains in the whey. Some proteoses-peptones are still allergenic, like caseinomacropeptide (16, 37), a fact which explains why allergic reactions may be observed after ingestion of whey protein hydrolysates by babies having positive RAST to casein but a negative one to whey proteins.

Table 2. Reactivity of isolated peptides obtained after chemical cleavage and enzymatic hydrolysis of β-casein against different classes of anti-bovine β-casein antibodies (adapted from Wal [68])

β-Casein-derived peptide	Rabbit IgG	Human IgG	Human IgE
1–25	+	–	–
1–60	+	–	+
26–60	+	–	–
61–93	+	–	–
29–93	+	–	+
94–102	+	–	–
103–109	+	–	–
110–144	+	–	+
132–144	–	–	+
157–185	+	+	+
186–209	–	+	+

The four caseins which constitute the whole-casein fraction are present in the milk of four ruminant species (cow, buffalo, sheep, and goat) with high sequence homologies ranging from 80% to more than 90%. This could lead to adverse reactions in patients allergic to cow's milk who consume milk from other ruminants (65). This casts much doubt on the suggestion that allergic reactions can be avoided by using milk from species other than cow and on the continuing use of goat's milk as a substitute for cow's milk. However, the IgE response may also be quite specific, with manifestations occurring after ingestion of sheep and goat cheese, and not with cow's milk or dairy products (66, 67).

Conclusions

The main characteristic emerging from all these studies is the multiplicity and diversity of allergens and, for each allergen, of the molecular immunoreactive structures (e.g., "epitopes") that are involved in CMA. 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. However, the structure of cow's milk allergens displays some features that could be common to food allergens.

Polysensitization to several proteins most often occurs; it is observed in about 75% of patients with CMA, with a great variability of the IgE response in both specificity and intensity. Even if the proteins that are most frequently and most intensively recognized by IgE seem also to be the most abundant in milk, e.g., the casein fraction and β-lactoglobulin, all milk proteins appear to be potential allergens, even those that are present in milk in trace amounts (e.g., Lf, Igs) and that are sometimes the only ones to be recognized as responsible for the clinical symptoms observed.

A high diversity also occurs in immunoreactive molecular structures. In each allergen, regions specifically recognized by IgE appear to be numerous and widely spread along the protein molecule. Moreover, these "allergenic epitopes", when they have been characterized, are often located in hydrophobic parts of the molecule, inaccessible to antibodies in the native conformation of the protein. Thus, human IgE seems to recognize denatured proteins and fragments better than the native ones. This high specificity to a denatured part of the protein probably reflects a specific mechanism of food allergy. Stimulation of the immune system and IgE response induction take place after gastric and intestinal digestion of the protein and then absorption and processing of the fragments. Therefore, the immunostimulating fragments can be different from those present and exposed on the native structure. It must therefore be noticed that the classical predictive tools for antigenicity based either on analysis of structure or on the antibody response in hyperimmunized animal models may sometimes be irrelevant for food allergens. Indeed, sequences recognized with a high frequency by human IgE are not always located in well-exposed hydrophilic regions of the molecule nor in regions in which structure is the most divergent from that of the human corresponding protein. Thus, a high degree of homology with the human corresponding sequence does not seem to prevent fragments from allergenic reactivity or at least from immunologic cross-reactivity with IgE.

Moreover, three-dimensional structure is an important feature in CMP allergenicity, but, besides the conformational epitopes, IgE-binding studies also showed the presence of sequential epitopes. These linear epitopes are peptides of various size depending on the method used to isolate and purify them, but, in some patients, peptides as short as 12–14 amino-acid residues have been characterized to account for a significant part of the allergenicity of the whole molecule. This observation justifies the need for caution before using peptides for hyposensitization or proposing any milk protein hydrolysate in the diet for highly allergenic children.

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