
Bovine milk allergenicity

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Objective: To provide updated data on the characteristics (eg, structure, function, stability) of the main milk proteins identified as allergens and on the characterization of their epitopes.

Data Sources: Basic literature and the most relevant original recent publications on clinical and epidemiologic aspects of milk allergy and the biochemistry and immunochemistry of milk proteins.

Study Selection: The expert opinion of the author was used to select the relevant data for the review.

Results: Most milk proteins, even proteins present at low concentrations, are potential allergens. Epitopes on milk proteins are both conformational and linear epitopes, widely spread throughout the protein molecules. They may be short fragments, located in hydrophobic parts of the molecule, that comprise highly conserved sequences responsible for IgE cross-reactivity with corresponding milk proteins of other mammals, including humans. Those sequential epitopes have also been proposed as good markers of persistent allergy to milk proteins and may be of particular clinical significance.

Conclusions: No specific structure or function is associated with allergenicity of milk proteins. Due to the great variability and heterogeneity of the human IgE response, no single allergen or particular structure can account for a major part of milk allergenicity. Furthermore, the available evidence is not sufficient to establish an intake threshold below which allergic reactions are not triggered or to predict reliably the effect of food processing on allergenic potential of milk proteins.

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INTRODUCTION

Milk allergy is an adverse reaction to milk proteins of different mammalian species, including cow, goat, and ewe, which is mediated by immunological mechanisms. It is mainly IgE mediated, but non-IgE-mediated reactions, which may involve other immunoglobulins, immune complexes, and/or cell-mediated mechanisms, also occur. It should be clearly distinguished from nonimmunological adverse reactions, such as lactose intolerance, which is due to lactase deficiency that occurs in large sections of the general population.^{1,2} Most IgE-mediated milk allergy appears in young children in the first 6 months of life and in most cases spontaneously disappears. The clinical picture can vary from mild-to-severe reactions that involve the skin, respiratory tract, or gastrointestinal tract and systemic reactions (anaphylactic shock).

Milk is one of the most common and widespread allergens that first affect atopic children in early life. It is an allergenic food for most populations. Data on prevalence of milk allergy are numerous and greatly vary, depending on the characteristics of the population studied (eg, geographic origin, atopic status, age) and the criteria used to identify the allergy (eg, self-reported allergy or diagnosis of allergy confirmed using in vitro or in vivo testing). Many studies in the general population have involved a great number of unselected children or adults.

In children, the prevalence of milk allergy ranges from approximately 1% to 2% to 5% or even 7.5%.^{3–9} It is note-

worthy that figures obtained through challenge tests or elimination (and thus confirming a clinical allergy) are generally 2 to 3 times lower than those recorded from questionnaire or in vitro serologic tests (eg, radioallergosorbent tests). Therefore, 2% appears to be a general mean figure for the prevalence of milk allergy in the general population of children. In unselected adults, the prevalence among the general population has been reported to be approximately 1%.^{10–12} According to numerous studies, a variable but large proportion of sensitive children spontaneously recover from their allergy between 2 and 5 years of age. Most children tolerate cow's milk at 3 years of age.^{13,14}

This study was performed to provide updated data on the characteristics (eg, structure, function, stability) of the main milk proteins identified as allergens and on the characterization of their epitopes. This review represents a synthesis of basic literature and the most relevant original recent publications on clinical and epidemiologic aspects of milk allergy and the biochemistry and immunochemistry of milk proteins. The expert opinion of the author was used to select the relevant data for the review.

MILK ALLERGENS

Structure, Function, and Allergenic Properties of Main Milk Proteins

Milk composition changes during lactation. It is noteworthy that milk of ruminant species other than cow (eg, buffalo, sheep, goat, humans, and many other species) is constituted by the same or at least very homologous proteins, which share the same structural, functional, and biological properties and are associated in more or less similar proportions (Table 1). However, human milk does not contain β -lactoglobulin (BLG).

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Table 1. Protein Composition of Milk of Different Mammalian Species

	Protein, g/L			
	Cow	Goat	Ewe	Mare
Whole casein	28–30	25–30	50–60	13
α_s -casein	14	2–6	25	
β -casein	11	18	25	
κ -casein	4	4	10	
Whey proteins	6	4	9	13

Cow's milk contains approximately 30 to 35 g/L of cow's milk proteins (CMPs). The action of chymosin (rennin) or the acidification of the milk to pH 4.6 enables 2 fractions to be obtained: lactoserum (whey), which constitutes approximately 20% of the CMPs, and coagulum (curd), which constitutes approximately 80% of the CMPs. Whey contains essentially globular proteins. The major ones, BLG and α -lactalbumin (ALA), are synthesized in the mammary gland, whereas others, such as bovine serum albumin (BSA), lactoferrin, and immunoglobulins, come from the blood. In the coagulum, the casein fraction comprises 4 proteins coded by different genes carried on the same chromosome: α_s1 -, α_s2 -, β -, and κ -caseins.

The main characteristics of the major milk proteins are presented in Table 2.^{15,16} The main characteristics that should be emphasized are the multiplicity and diversity of proteins that are involved in cow's milk allergy (CMA). Polysensitization to several proteins most often occurs, and all milk proteins appear to be potential allergens. Studies of large populations of allergic patients show that most of the patients are sensitized to several proteins, including BLG (Bos d 5), casein (Bos d 8), ALA (Bos d 4), BSA (Bos d 6), lactoferrin, and immunoglobulins (Bos d 7).^{17–25} A great variability is observed in IgE response. Both casein and BLG, as well as ALA, are major allergens. However, proteins present in very low quantities, such as BSA, immunoglobulins, and especially lactoferrin, also appear to be important, since 35% to

50% of patients are sensitized to those proteins and sometimes to those proteins only.²³ In the last few years, sensitivity to casein has increased in terms of both frequency and intensity of IgE response.^{16,25} Sensitizations to casein, BLG, and ALA are closely linked. In contrast, sensitivity to BSA appears to be completely independent, with 50% of the patients being allergic to BSA regardless of their sensitivity to other milk allergens.²⁴

The primary structure and part of the secondary and tertiary structure of CMPs are known. However, caseins cannot be crystallized, and their tertiary structure is approached by molecular modeling. The major milk allergens present in lactoserum (whey) are BLG and ALA, but proteins present at a very low concentration may also be important allergens, and lactoferrin is an interesting example.

BLG (Fig 1) 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 2 disulfide bridges and 1 free cysteine. This structure is responsible for the main physicochemical properties and also for interaction with casein during heat treatments. The relative resistance of BLG to acid hydrolysis and gut proteases allows part of the protein to be absorbed intact through the intestinal mucosa. The tertiary structure of BLG is known (Fig 1B). It belongs to the lipocalin family and is considered a retinol-binding protein. Lipocalins have a high allergenic potential, and several allergens of animal origin belong to this family. They share well-conserved sequence homologies in their N-terminus moiety, where tryptophan at position 19 is always present, despite the mutations that have occurred during evolution. Crystallography studies revealed very similar folding, called β -barrel structure, with the same arrangements of 8 (or 10) antiparallel β -sheets.^{26–32}

ALA (Fig 2) is a monomeric globular protein of 123 amino acid residues with a molecular weight of 14.4 kDa and 4 disulfide bridges. It is a regulatory component of the enzymatic system of galactosyl transferase responsible for the synthesis of lactose. It possesses a high-affinity binding site

Table 2. Main Characteristics of the Major Bovine Milk Proteins

Protein concentration	Concentration, g/L	Molecular weight, kDa	No. of amino acid residues per molecule
20% Whey (approximately 5 g/L)			
10% BLG (Bos d 5)	3–4	18.3	162
5% ALA (Bos d 4)	1–1.5	14.2	123
3% Immunoglobulins Bos d 7	0.6–1.0	150	...
1% BSA (Bos d 6)	0.1–0.4	66.3	582
Traces of lactoferrin	0.09	80	703
80% Whole casein (Bos d 8) (approximately 30 g/L)			
32% α_s1 -casein	12–15	23.6	199
10% α_s2 -casein	3–4	25.2	207
28% β -casein	9–11	24.0	209
10% κ -casein	3–4	19.0	169

Abbreviations: ALA, α -lactalbumin; BLG, β -lactoglobulin; BSA, bovine serum albumin.

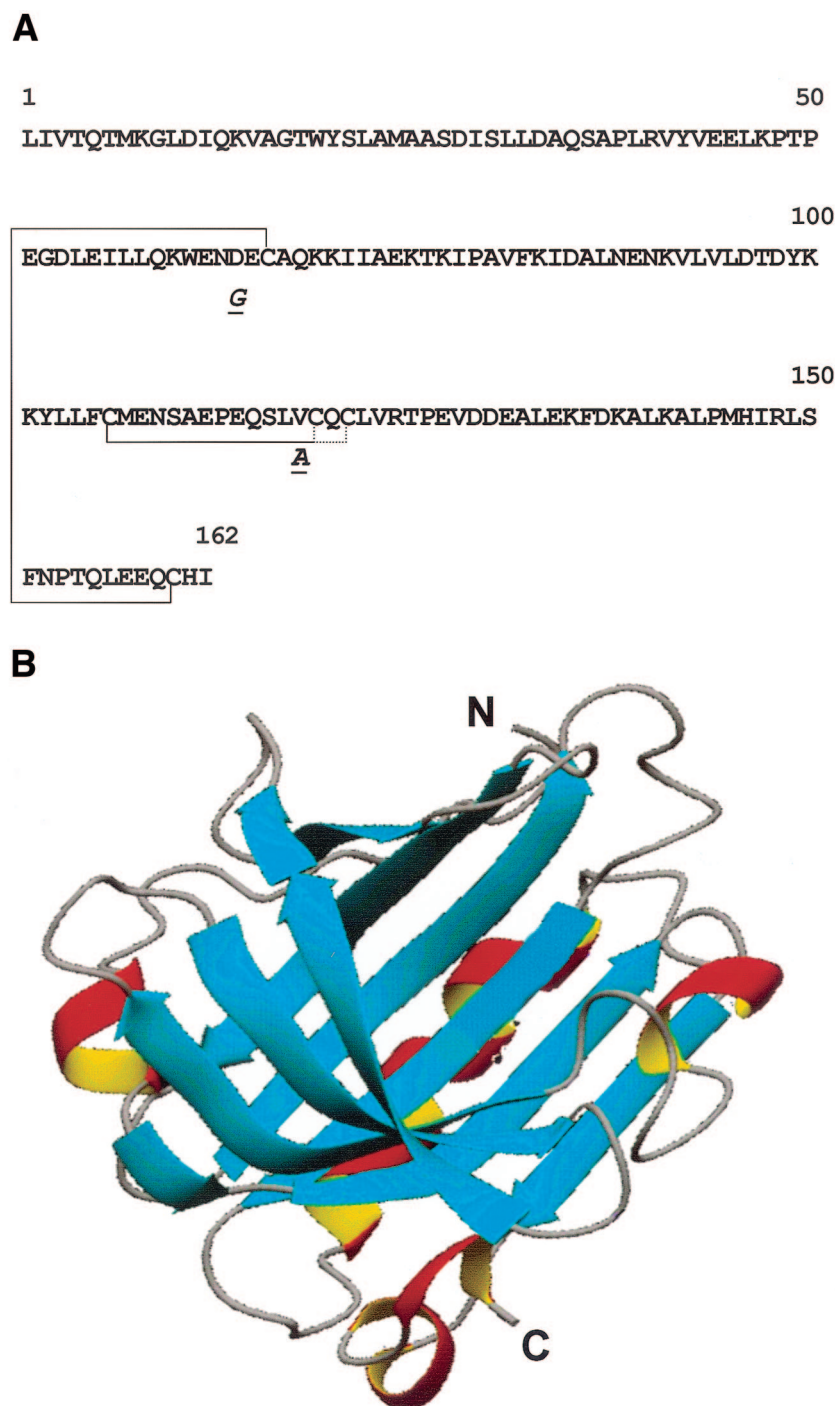


Figure 1. A, Amino acid sequence and location of disulfides bridges of β -lactoglobulin variant A and variant B. B, Three-dimensional structure of β -lactoglobulin.

for calcium, and this bond stabilizes its secondary structure. The complete amino acid sequence of bovine ALA shows extensive homology with hen's egg white lysozyme but also with human ALA.^{33–36}

Lactoferrin (Fig 3) is an iron-binding glycoprotein of the transferrin family. It is present in very low concentrations in cow's milk, but it has been shown to be an important allergen.¹⁶ It is present in much higher concentrations in human

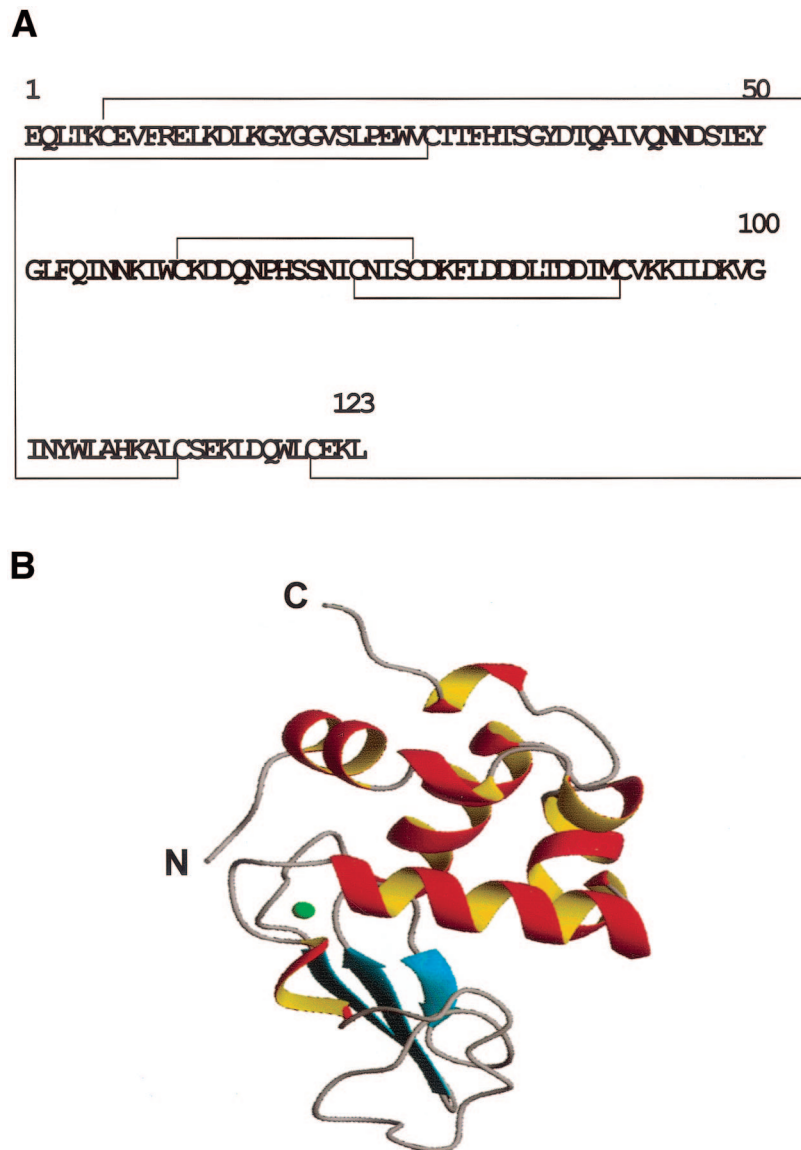


Figure 2. A, Amino acid sequence and location of disulfides bridges of α -lactalbumin. B, Three-dimensional structure of α -lactalbumin.

breast milk (ie, 1 g/L) but particularly in colostrum. Human and bovine lactoferrin have high amino acid sequence homology (69%) and structure similarity. The 3-dimensional structure is known and presents 2 lobes, each of them having a high-affinity iron-binding site.³⁷ Lactoferrin is partially heat stable and relatively stable to enzymatic degradation by gut proteases and remains partly unchanged during digestion. Its main biological function is to act as an antioxidant and a scavenger for free radicals, thus providing a protection against oxidative stress. It also has antibacterial properties and has been shown to stimulate cellular immune defense of the organism against infections.

Whole casein fraction constitutes the coagulum (ie, the solid fraction of proteins obtained after coagulation of milk).

Each individual casein, α_s1 -, β -, α_s2 -, and κ -casein, represents a well-defined chemical compound, but they cross-link to form ordered aggregates (ie, micelles) in suspension in lactoserum (whey). Their proportion in the micelles is relatively constant at approximately 37%, 37%, 13%, and 13%, respectively. Their distribution is not uniform within these micelles, which comprise a central hydrophobic part and a peripheral hydrophilic layer, where major sites of phosphorylation that contain phosphoserine residues are presented, in relation to the calcium-binding and transfer properties of caseins. α_s1 -, α_s2 -, β -, and κ -casein have little primary structure homology. Their functional properties also differ, since 3 of them, α_s1 -, α_s2 -, and β -casein, appear to be calcium sensitive, whereas κ -casein is not. However, the 4

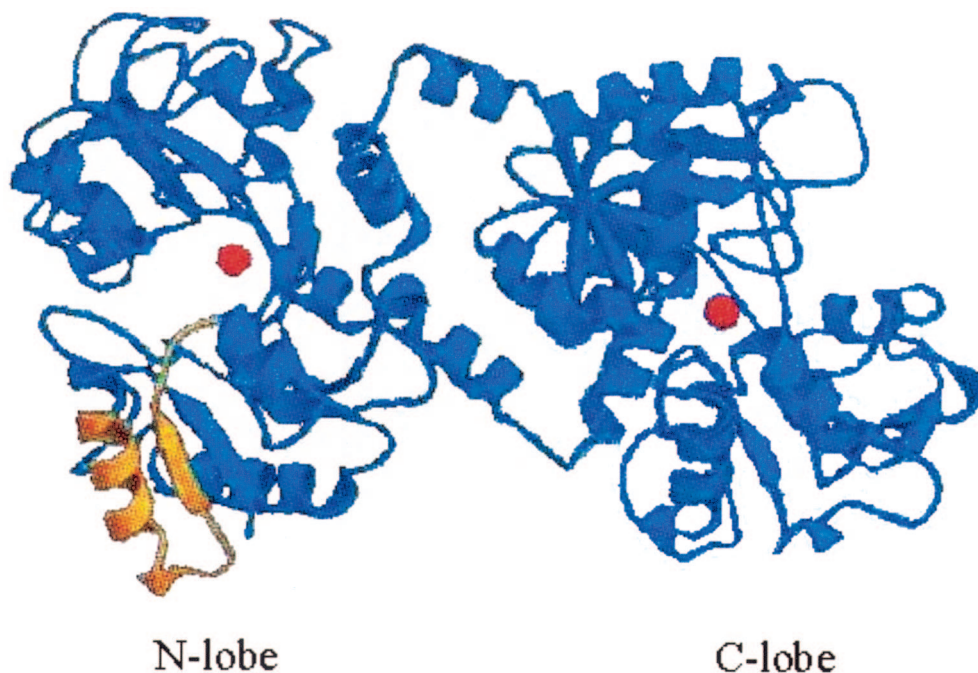


Figure 3. Three-dimensional structure of lactoferrin.

caseins display common features that are unusual, differing greatly from other milk proteins. They are phosphorylated proteins, with a loose tertiary, highly hydrated structure.³⁸ Casein is often considered poorly immunogenic because of this flexible, noncompact structure and because it is rapidly and extensively degraded by proteolytic enzyme during digestion. Caseins are not significantly affected by severe heat treatments but are very susceptible to all proteinases and exopeptidases. Multisensitizations to the different caseins most often occur in patients sensitized to the whole casein fraction.³⁹

Several forms of caseins have been described that derive from partial hydrolysis that naturally occurs in milk (eg, by plasmin). Several of such peptidic fragments derived from caseins conserve part of the allergenicity of the native protein.

Due to the great variability of human IgE response, no single allergen or particular structure can account for a major part of milk allergenicity. Polysensitization to several proteins most often occurs; it is observed in approximately 75% of patients with CMA, with a great variability of the IgE response both in specificity and intensity. Even if the proteins most frequently and most intensively recognized by IgE seem also to be the most abundant in milk, casein, and BLG, all milk proteins appear to be potential allergens, even those that are present in milk in trace amounts (eg, lactoferrin). These are sometimes the only ones to be recognized and responsible for the clinical symptoms observed. In such cases, identification of the responsible allergen for CMA requires specific and sensitive immunochemical tests.

CMPs are heterogeneous, with few structural or functional common features. This heterogeneity is complicated by their genetic polymorphism, resulting in several variants for each protein. These variants are characterized by point substitutions of amino acids, deletions of peptide fragments of varying size, or posttranslational modifications such as phosphorylation and glycosylation. All of these modifications and similar modifications that occur throughout processing may affect the IgE-binding capacity and allergenicity.^{40,41}

Characterization of Epitopes on CMPs

No definite relationship can be established between structure and allergenicity. It appears that the 3-dimensional structure is an important feature in CMP allergenicity, but besides conformational epitopes, IgE-binding studies also show the presence of sequential epitopes. These linear epitopes are peptides of various size, depending on the method used to isolate and purify them, but peptides as short as approximately 12 to 14 amino acid residues (ie, approximately 1,500-Da molecular weight) have been demonstrated to account for a significant part of the allergenicity of the whole molecule in some patients.

Sensitization has been shown to involve many continuous epitopes that are widely spread throughout the BLG molecule by using tryptic and synthetic peptides^{42,43} or overlapping peptides.⁴⁴ The best recognized peptides, by more than 90% of the patients, are BLG fragments (41–60), (102–124), and (149–162), each accounting for 10% to 15% of the whole BLG immunoreactivity.

IgE binding to native ALA and large peptides confirmed the importance of conformational epitope(s). However, in several patients, reduced and denatured peptides exhibited a similar or higher IgE-binding capacity than the native corresponding fragment, suggesting the existence of sequential epitope(s) exposed through protein denaturation. Furthermore, IgE-binding sequences are also located in strongly hydrophobic regions of the ALA molecule, where antigenicity is very unlikely to be predicted, and/or within parts of the molecule that have a high sequence homology with human ALA.^{45,46}

Jarvinen et al,⁴⁷ using overlapping decapeptides, identified numerous IgE- and IgG-binding epitopes of BLG and ALA. They confirmed the variability of the antibody responses to various regions of the molecules. Interestingly, the authors correlated the presence of IgE to multiple linear epitopes with persistent (vs transient) milk allergy and suggested it could be a good marker to identify the patients that would have a lifelong milk allergy. The incidence of the conformation of the allergen on the nature of the clinical manifestations that occur during the allergic reaction to BLG has been evidenced by Adel Patient et al,⁴⁸ using a mouse model of milk allergy. Mice sensitized to native BLG were challenged by either native or denatured BLG. In the last case, only linear epitopes were present and immediately bioavailable, whereas conformational epitopes were present on native BLG. Depending on the structure of the allergen, 2 alternative mechanisms of mast cell activation, of different intensities, may be triggered

with consequences on both the early and late phases of the reaction. They specifically involved either peptido-leukotrienes or prostaglandin D₂ in case of challenge by native or denatured BLG, respectively.

As mentioned previously, most patients allergic to casein are sensitized to each of the 4 caseins. This likely results from a cosensitization to the different casein components after disruption of the casein micelles during the digestive process. However, polysensitization also appeared to be due to cross-sensitization mechanisms and involved the only conserved regions that contain the major sites of phosphorylation. Interestingly, bovine β -casein induces a high IgE response despite the fact that it is also abundant in human milk and that human and bovine β -caseins share a high sequence homology. It has been shown that conserved regions shared by both bovine and human β -caseins and particularly those comprising clusters of phosphorylated seryl residues are responsible for IgE cross-reactivity^{39,49} (Fig 4). Furthermore, it is noteworthy that some of the major epitopes already characterized on α_s -caseins are continuous epitopes that have also been located in hydrophobic regions of the molecule, where they are not accessible to antibodies unless the casein is denatured or degraded, such as, for instance, during digestion.^{50,51} In addition, Chatchatee et al⁵¹ have observed differences in the anti-casein IgE capacity to bind different epitopes of α_s 1-casein between 2 groups of patients with persistent or transient CMA. Sixty-seven percent and 100% of patients older than 9 years with persistent allergy specifically recognized

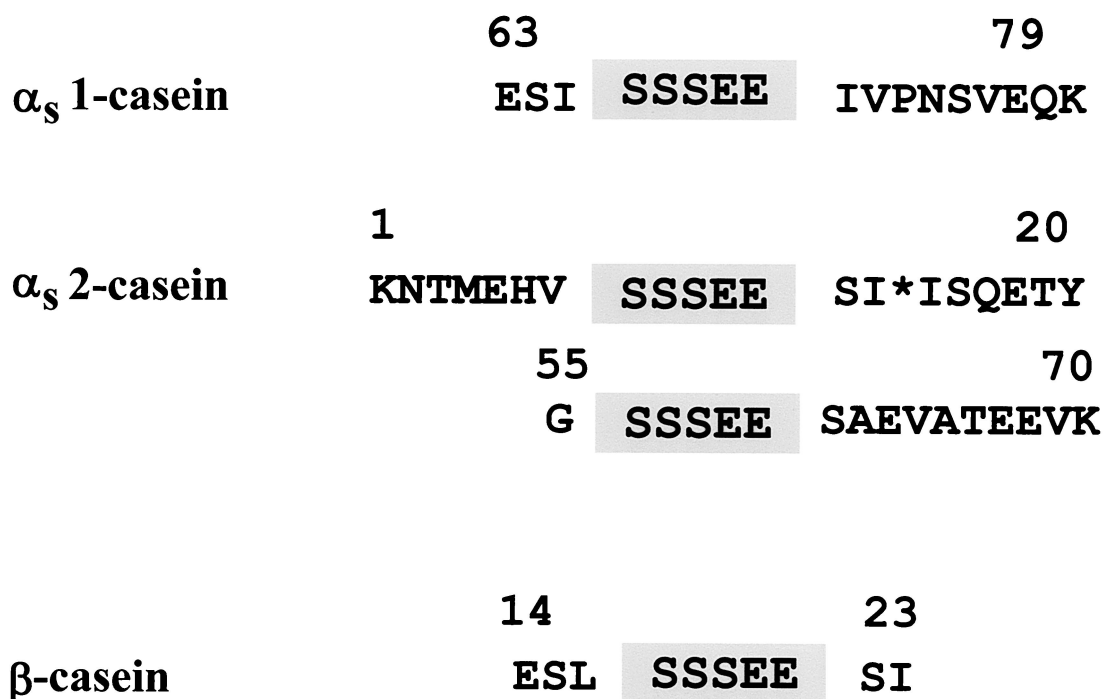


Figure 4. Major sites of phosphorylation in caseins.

sequences (69–78) and (173–194), respectively, whereas none of the children younger than 3 years, who are likely to outgrow CMA, had specific IgE to those fragments. In addition, sequence (69–78) was not found to be an IgG-binding epitope in any group. Such a relationship between CMP epitope sensitization and natural history of CMA is a relevant topic in clinical practice^{52,53}

Several T-cell epitopes have also been characterized, and sequence (101–112) appears to be the core sequence in the peptides that most frequently interact with HLA molecules in the immunoregulation of T-cell responses to BLG.⁵⁴

Cross-reactivities

The same or closely homologous proteins and their variants are present in milk of other ruminant species. Of particular importance is the fact that the same caseins are present, with high sequence homologies varying from 80% to more than 90%. As a consequence, a high IgE cross-reactivity among ewe's, goat's, and cow's milk casein occurs in most patients with CMA.^{55–58} However, the IgE response may also be specific, with allergic reactions to goat's and ewe's milk without associated CMA⁵⁹ and manifestations that occur after ingestion of sheep's and goat's cheese but not cow's milk or other dairy products.⁶⁰ Table 3, adapted from Ribadeau-Dumas,⁶¹ shows the sequence homology between the main proteins of cow's, goat's, and ewe's milk.

Cross-reacting allergens other than proteins from milk of other species may also exist. Adverse reactions have been reported in milk allergic patients fed soy-based formulae as cow's milk substitutes. A 30-kDa, glycinin-like protein from soybean that cross-reacts with cow's milk casein has been isolated and partially sequenced.⁶²

Possible Effects of Processing on Allergenicity

These considerations on the structure and properties of CMPs and on the structure and location of their IgE-binding epitopes, particularly the evidence of existence and importance of linear epitopes, have implications on the incidence of technological and physiologic processing on milk allergenicity. Heat processing has no definite or unequivocal impact on CMP allergenicity. Casein is thermostable, whereas BLG is

thermolabile, but it may be protected through interaction with casein. Therefore, the results depend not only on temperature and time of heating but also on possible interactions within the food matrix. Heat denaturation, which leads to the loss of organized protein structures, does not always result in a decreased allergenic potential. On the contrary, formation of aggregates may increase the allergenicity of the heated product. When the treatment results in a decrease of the allergenicity, it is always limited. As examples, boiling of milk for a few minutes (2, 5, or 10 minutes) results either in no difference or in a reduction of approximately 50% to 66% of the positive reactions compared with raw milk; similar observations have been reported with raw vs pasteurized or homogenized and pasteurized milk.^{19,63–65}

It is generally accepted that the hydrolysis of milk proteins considerably reduces their allergenicity. However, several studies have shown that specific IgE from patients with milk allergy recognized enzymatic digestion products of whey proteins (eg, BLG and ALA) or casein and that the recognition of peptides may even be better than that of the intact molecule.^{43,45,50,66,67} Several studies have reported controversial results with hydrolyzed formulae, depending on the enzymes used and the degree of hydrolysis. As mentioned, some CMPs are resistant to degradation by proteolytic enzymes (eg, BLG), whereas others are considered very labile (eg, casein). The incidences of reported adverse effects on infants fed partially or extensively hydrolyzed milk (either casein or whey) formulae range from approximately 45% to 65% and 15%, respectively.^{68–70} For partially hydrolyzed formulae, allergic reactions may be due to the presence of some residual native protein or of large fragments derived therefrom. For extensively hydrolyzed formulae, where no protein or large fragments are still present, the allergic reaction may be triggered by short peptidic fragments that comprise the IgE-binding epitopes, such as those described herein, that are released during the proteolysis.

Threshold Doses

As for other food allergens, there are few data on thresholds of eliciting doses obtained through well-documented clinical studies using food challenges. Those studies are rare and biased, because testing patients known to be highly reactive is risky and might even be considered unethical due to the severity of clinical manifestations that may occur. However, indirect indications on such threshold doses have been indirectly obtained from reports of severe adverse reactions, including anaphylactic shocks, that occurred after ingestion of minute amounts of dairy products, such as cheese. In addition, such adverse reactions have also been reported after ingestion of foods that contain hidden CMPs in which the amounts of contaminating CMPs have been afterward quantified. They include foods that may be considered *nondairy products* but that contain residual amounts of CMPs used as processing aids. They also include breast milk from mothers who have eaten products made from cow's milk. Indeed, BLG from ingested cow's milk is absorbed through the gut

Table 3. Comparisons of Amino Acid Sequences of Main Proteins of Cow's, Goat's, and Ewe's Milk*

	Sequence homology, %		
	Cow vs goat	Cow vs ewe	Goat vs ewe
BLG	96	96	99
ALA	95	94	99
Casein			
α_s1	87	89	97
α_s2	88	89	98
β	90	90	99
κ	85	84	95

Abbreviations: ALA, α -lactalbumin; BLG, β -lactoglobulin.

* Adapted from Ribadeau-Dumas.⁶¹

mucosa and can be detected in human milk; it may be responsible for colic in breast-fed infants.^{71–73} In reports where breast-fed infants experienced severe reactions, the concentrations of contaminating CMPs were as low as 0.5 to 50 ng/mL, and reactions often reportedly occurred at approximately 5 ng/mL.^{28,74–77} Bindslev-Jensen et al⁷⁸ proposed a statistical approach to determine threshold levels from published data in the literature and then derive an acceptable intake for a given proportion of an allergic population (eg, 90%), using a paradigm similar to that used in classic toxicology. Until now, the recorded data show that a substantial part of the population reacts to very low (in the range of micrograms) amounts of allergens, similar to those reported in cases of adverse reactions.

Severe reactions have also been reported after ingestion of meat products that contain casein used, for example, as a texturing agent at concentrations ranging from 1.1% to 0.04%.⁷⁹ Frozen desserts that contain unexpected trace amounts of whey proteins (9 µg/mL) likely due to cross-contamination at the factory during manufacture have provoked anaphylaxis in a 3-year-old boy.⁸⁰ Food-quality lactose, which can be present in baby food expected to be free of CMPs, may contain trace amounts of CMPs sufficient to cause failure to thrive, digestive troubles, and coughing.⁸¹ Anaphylactic reaction to lactose-containing medications that have been contaminated by milk protein has also been recently reported.⁸²

CONCLUSION

Milk is a frequent and a major allergenic food. Most CMPs are potential food allergens, even those present at low concentrations. Numerous sequential epitopes have been identified that are widely spread throughout the protein molecules. Short peptidic fragments may conserve a significant part of the allergenicity of the whole protein. Some are located in hydrophobic parts of the molecule and are released or become bioavailable after denaturation of the proteins or after their degradation by proteolytic enzymes, for example, during digestion. Any milk product that contains native or denatured CMPs or fragments derived thereof may trigger an allergic reaction, even those present in nondairy foods, such as cross-contaminant or processing aids. Previous studies have shown that allergic reactions can be triggered by small amounts of CMPs (in the range of micrograms). Data available from controlled food challenges or case reports do not permit the establishment of validated threshold doses or the determination of a level of exposure that could protect allergic consumers against a reaction to milk products present in their food. There is no definite indication that technological treatments may alter the structure of CMPs or decrease their concentration to a sufficient extent to guarantee the loss of their allergic potential, at least for a highly sensitive fraction of the population of milk sensitive patients. Furthermore, due to the high frequency of immunological cross-reactions, these considerations may be applied to milk of species other than cows, such as buffalo's, goat's, and ewe's milk.

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