

Cross-reactivity between mammalian proteins

Patrizia Restani, PhD*; Barbara Beretta, PhD*; Alessandro Fiocchi, MD†; Cinzia Ballabio, PhD*; and Corrado L. Galli, PhD*

Background: Cross-reactivity between food allergens occurs when they share part of their amino acid sequence, or when their three-dimensional molecular structure causes them to have a similar capacity to bind specific antibodies.

Objectives: To review data from our laboratory on cross-reactivity between mammalian proteins (milk and meat allergens).

Methods: Studies used immunoelectrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis/polyacrylamide gel electrophoresis and immunoblotting), and animal monoclonal antibodies.

Results: The findings suggest that animal monoclonal antibodies specific for cow's milk proteins are able to recognize the major part of milk proteins from mammals bred in Mediterranean countries (sheep, goat, and buffalo); weak cross-reactivity was observed with milk proteins from mares and donkeys. None of the antibodies used in our studies reacted with proteins from an exotic mammalian species: the camel. Similar cross-reactions were found with human circulating immunoglobulin E from children allergic to milk. With regard to beef allergy, monoclonal antibodies specific for bovine serum albumin cross-reacted only with ovine serum albumin, whereas the number of sera from allergic children able to recognize other mammalian serum albumins depended directly on the closeness of phylogenetic relationship between animal species and inversely on the percent identity with human serum albumin in the main epitopic sequence.

Conclusion: An area of heterogeneity between animal and human species in a critical amino acid sequence (epitope) of an allergen can determine the degree of immunogenic activity.

Ann Allergy Asthma Immunol 2002;89(Suppl):11-15.

INTRODUCTION

Allergic reactions to mammalian proteins present in foods are quite common, although their actual incidence is uncertain. Allergy to cow's milk and beef is the most frequent adverse reaction to foods of animal origin, but cases of hypersensitivity to milk and meat from other mammalian species (goat's milk, pork meat, etc) have been described.

Cross-reaction occurs when two food proteins share part of the amino acid sequence (at least, that containing the epitopic area) or when the three-dimensional conformation makes two molecules similar in their capacity to bind specific antibodies. In general, cross-reactivity reflects the phylogenetic relations between animal species: homologous proteins from vertebrates are often cross-reactive.¹

Cow's milk contains several proteins known to be potential antigens; they can be generally classified in two major groups: caseins and whey proteins. Because β -lactoglobulin is absent from human milk, it has long been considered the most important of cow's milk allergens,^{2,3} but several studies have demonstrated that the casein fraction also has important antigenic potential.^{4,5} Goat's milk can be tolerated by some children who are allergic to bovine milk,^{6,7} yet there is strong evidence of cross-reactivity between cow's and other milk proteins.^{8,9}

Meat allergy has long been considered rare, occurring mainly in children, but studies performed by our group¹⁰⁻¹² showed that beef allergy can occur quite frequently (9% in 113 atopic children). Bovine serum albumin was identified by skin prick test, immunoblotting, and double-blind, placebo-controlled food challenge as one of the most important allergens in beef,¹⁰⁻¹³ along with immunoglobulins. Both these types of protein are involved in the cross-reactivity between meats from different animal species and in milk/meat co-sensitization.

We have reviewed our data on cross-reactivity between milk/meat proteins from different animal species, in the hope of obtaining new insights into the molecular basis of allergic reactions and also of identifying more suitable protein sources for allergic children. A good alternative diet for children allergic to cow's milk has not yet been found. The substitution of soy derivatives and hydrolyzed protein formulas for cow's milk is not free of risk for sensitized children. In fact, 10 to 20% of children allergic to cow's milk do not tolerate soy,¹⁴ and some cases of anaphylactic reaction to extensively hydrolyzed formulas have been reported.^{15,16}

MATERIALS AND METHODS

Milk Samples

Buffalo, cow's, and ewe's milk were kindly supplied by cheesemakers around Brescia, camel's milk by Roberta Lodi, Consiglio Nazionale delle Ricerche, Milk Center, Milan; mare's, donkey's, and mule's milk by Stefania Iametti, Dipartimento di Scienze Molecolari Agroalimentari, University of Milan. Human milk was from a volunteer donor. The milk samples were diluted with distilled water 1:4 (vol:vol); the

* Laboratory of Toxicology, Department of Pharmacological Sciences, University of Milan, Milan, Italy.

† Pediatric Department, Macedonio Melloni Hospital, Milan, Italy.

Received for publication February 22, 2002.

Accepted for publication in revised form July 2, 2002.

obtained samples were treated with sample buffer (0.25 M Tris-HCl buffer, pH 6.8, containing 7.5% glycerol, 2% sodium dodecyl sulfate [SDS], 5% β -mercaptoethanol) in the ratio 1:1 (vol:vol). Samples were heated for 10 minutes at 100° C and loaded onto an electrophoresis gel.

Purified Serum Albumins

Beef (BSA), chicken, horse (HoSA), human (HSA), pig (PSA), rabbit (RSA), sheep (OSA), and turkey serum albumins were purchased by Sigma Aldrich (Milan, Italy) at the highest available purity. Serum albumins were solubilized at a final concentration of 1 mg/mL in native sample buffer (0.25 M Tris-HCl buffer, pH 6.8, containing 7.5% glycerol).

Polyacrylamide Gel Electrophoresis (PAGE)

Samples were separated according to Restani et al.^{5,11} on a polyacrylamide gradient gel (9 to 19% of acrylamide) without (PAGE) or with (SDS-PAGE) SDS as a denaturing agent. After the electrophoresis run (90 V at room temperature, for approximately 6 hours) the gels were dyed with Coomassie Brilliant Blue G-250.⁵

Immunoblotting

After PAGE or SDS-PAGE, immunoblotting was performed as described by Restani et al.⁵ The membranes were incubated overnight in 10 mL of 0.25% gelatin solution containing 10 μ L of murine monoclonal antibodies. The antigen-immunoglobulin (Ig)G complex was detected using goat anti-mouse IgG antibodies (Sigma Aldrich), labeled with alkaline phosphatase; the secondary antibody commercial stock was diluted 1:1000 (vol:vol) in 0.25% gelatin solution.

After incubation in bromochloroindolyl phosphate/nitroblue tetrazolium solution, an intense black-purple precipitate developed at the site of enzyme binding. The developing solution contained 15% bromochloroindolyl phosphate and 30% nitroblue tetrazolium in alkaline phosphatase buffer (100 mmol/L Tris, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, pH 9.5).

RESULTS

Milk Proteins

Milk proteins from different animal species were separated by SDS-PAGE; the patterns of different samples are shown in Figure 1. The use of SDS as a denaturing agent is necessary because milk is a complex matrix and its electrophoretic separation under non-denaturing conditions is unsatisfactory.

In cow's milk, the four major bands correspond to α -casein, β -casein, β -lactoglobulin, and α -lactalbumin (from the top to the bottom of the gel). Buffalo milk showed a similar pattern, with a fifth band at the bottom and two other proteins with low electrophoretic mobility. Camel's milk showed a quite different pattern with a higher protein band in the area of caseins and the absence (or only traces) of β -lactoglobulin. Mare's milk contained two bands in the casein area (although with different electrophoretic mobility) and three proteins in the area of whey proteins. Donkey milk presents one major band in the area of casein and three proteins in the area of whey proteins. Mule milk, obtained from an animal pregnant after artificial insemination, was identical to mare's milk. Human milk, used to verify the specificity of reaction in immunoblotting, has a more complex protein pattern; in fact, in addition to caseins (β and κ fractions) and α -lactalbumin, it presents a band with high molecular weight (low electrophoretic mobility) corresponding to lactoferrin. The absence of β -lactoglobulin in human milk was confirmed.

Figure 2 shows the positive responses obtained by immunoblotting with an anti- β -lactoglobulin monoclonal antibody. Antibodies are able to recognize β -lactoglobulin from ewe, goat, and buffalo, although weak cross-reactivity was observed with mare's and donkey's milk proteins. No anti- β -lactoglobulin antibody (we tested four different monoclonal preparations) reacted with milk proteins from a camel.

A similar pattern of reactivity was observed using anticasein monoclonal antibodies and the sera of children allergic to cow's milk (see our previous paper, ref. 9, where cross-reactivity has

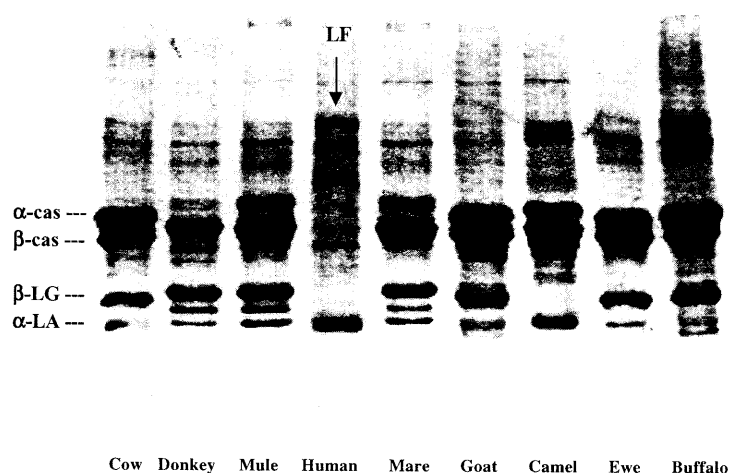


Figure 1. SDS-PAGE of milk samples from different animal species. α -cas, α -casein; β -cas, β -casein; α -LA, α -lactalbumin; β -LG, β -lactoglobulin; LF, human lactoferrin.

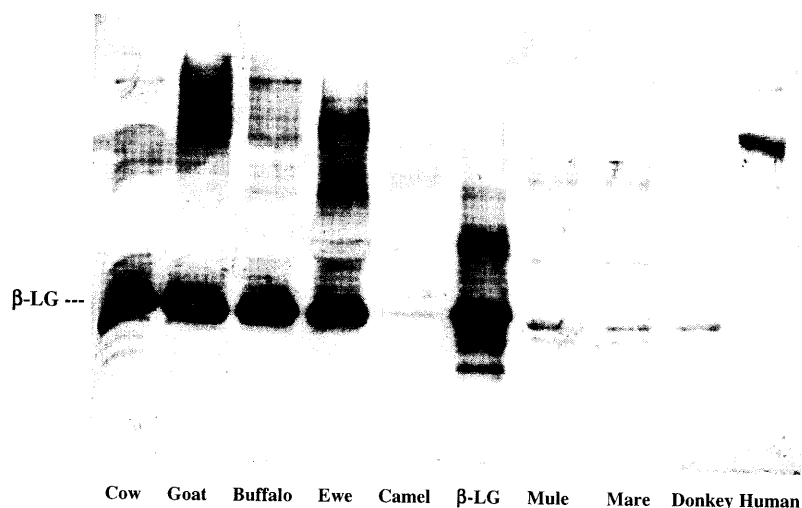


Figure 2. Immunoblotting of milk samples from different animal species obtained after incubation with an anti β -lactoglobulin monoclonal antibody. β -LG, β -lactoglobulin.

been studied using human IgEs). Homologies in amino acid composition could justify the cross-reactivity observed between proteins from different animal species. By contrast, phylogenetic differences could account for the failed recognition of camel's proteins by animal and human antibodies.

Meat Proteins

Serum albumins from different animal species can be separated by electrophoresis under non-denaturing conditions (PAGE; Fig 3) because serum albumins are easily soluble in non-denaturing buffer. Under non-denaturing conditions, the three-dimensional structure of the proteins is conserved. The electrophoretic mobilities of the different species' serum albumins are very similar, as was expected from their similar molecular weight. Two more bands, the dimer and the trimer of the same proteins, are visible in each electrophoretic pattern.

Figure 4 illustrates the immunoreactivity obtained by incubating membranes with a commercial anti-BSA monoclonal antibody. This recognized BSA and OSA with similar

affinity, but did not bind to other serum albumins. Any positive reaction always included the dimer and the trimer of serum albumins. These data confirm that cross-reactivity reflects the phylogenetic affinity of animal species.

As we have shown previously,^{11,17} human IgEs from children allergic to beef recognize bovine, ovine, and pig serum albumin, but not horse, rabbit, chicken or turkey serum albumins. Here too, homologies in amino acid composition could account for the cross-reactivity between serum albumins from different animal species.

The percentage of sequence identity between BSA and other serum albumins correlated well with their ability to bind specific anti-BSA antibodies (Table 1): OSA 92.4%, PSA 80.0%, HoSA 74.5%, RSA 72.2%, chicken serum albumin 44.7%. However, the fact that a small (5.5%) sequence difference between PSA and HoSA changes their binding to antibodies drastically led us to hypothesize¹² and then demonstrate¹⁶ that the BSA epitope is located in the area where the 5.5% sequence difference occurs.

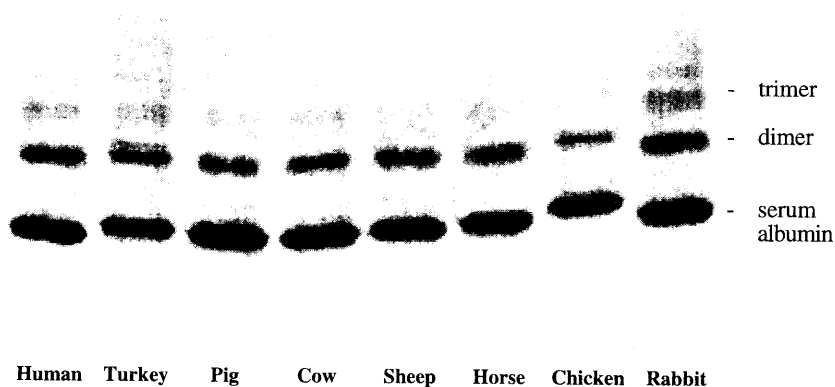
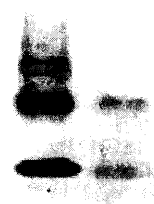


Figure 3. PAGE of serum albumin from different animal species.



- trimer
- dimer
- serum albumin

Figure 4. Immunoblotting of serum albumins from different animal species obtained after incubation with an anti-BSA monoclonal antibody.

Human Turkey Pig Cow Sheep Horse Chicken Rabbit

Table 1. Sequence Alignment of Serum Albumins from Mammalian Species and Percentage of Identity versus BSA and HSA (table modified from reference 17)

Animal species	Aminoacidic sequence*									Id. vs HSA ^a (%)	Id. vs HSA ^b (%)	Id. vs BSA (%)	Reacting sera (%)
	524	525	526	527	528	529	540	541	542				
Cow	A	F	D	E	K	L	P	D	T	76.3	57.8	100.0	100.0
Sheep	P	F	D	E	K	F	P	D	T	75.8	57.8	92.4	100.0
Pig	E	F	V	E	G	T	P	E	D	76.9	63.2	80.0	83.3
Horse	E	F	K	A	E	T	P	E	D	76.9	84.2	74.5	0.0
Rabbit	E	F	N	A	E	T	P	E	T	75.2	89.5	72.2	0.0
Human	E	F	N	A	E	T	S	E	K	100.0	100.0	76.3	0.0

Id. = percentage of identity (data on identity from reference 17)

* Number of amino acid is relative to BSA

^a Identity in the whole amino acid sequence

^b Identity in the identified epitopic area: aa 524–aa 542 (reference 18)

BSA, OSA, and PSA show an area (aa 526 to 529) very different from the corresponding sequence in HoSA (Table 1). The non-cross-reacting HoSA and RSA contain amino acids in this area that make them more similar to HSA.

By contrast, the percentage of sequence identity with HSA of a specific allergen seems not to correlate with the number of positive responses in immunoblotting (Table 1). In fact, all the mammalian albumins considered in this study revealed an overall percentage of identity with human protein close to 75%.

CONCLUSION

Milk Allergy

The use of goat's milk (an occasional clinical practice) should be avoided because of the high risk of cross-reactivity. Mare's and donkey's milks, used in popular practice for allergic children, are promising alternative protein sources when appropriately modified in chemical composition and evaluated from the hygienic point of view. Camel's milk

seems to be an even more interesting alternative if the problems of availability and technologic treatment (a new sensitization must be avoided) can be overcome.

Beef Allergy

Allergenic cross-reactivity between proteins from different mammalian species correlates with their phylogenetic affinity (Table 2). There is no inverse correlation between the aller-

Table 2. Phylogenetic Listings of Mammal Species Included in Meat Allergy Study

Order	Family	Animal species
Artiodactyla	Bovidae	Cow, goat, sheep
	Suidae	Pig
Perissodactyla	Equidae	Horse
Lagomorpha	Leporidae	Rabbits
Primates	Hominidae	Human beings

From *Mammals of the World*—online by Ronald Nowak (<http://www.press.jhu.edu>).

genic potential of a given protein and its percentage of amino acid sequence identity with the corresponding human sequence. However, a specific area of heterogeneity between animal and human species in the amino acid sequence of an allergen can determine its immunogenic activity. Antibodies from animal species do not always behave like human IgEs, so immunologic data obtained in experimental animals can not be directly extrapolated to humans.

ACKNOWLEDGMENTS

This research was supported by MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica).

REFERENCES

1. Spitzauer S. Allergy to mammalian proteins: at the borderline between foreign and self? *Int Arch Allergy Immunol* 1999;120:259–269.
2. Goldman AS, Sellars WA, Halpern SR, et al. Milk allergy. II Skin testing of allergic and normal children with purified milk proteins. *Pediatrics* 1963;32:572–579.
3. Gjesing B, Osterballe O, Schwartz B, et al. Allergen-specific IgE antibodies against antigenic components in cow milk and milk substitutes. *Allergy* 1986;41:51–56.
4. Savilahti E, Kuitunen M. Allergenicity of cow milk proteins. *J Pediatr* 1992;121:S12–S20.
5. Restani P, Velonà T, Plebani A, et al. Evaluation by SDS-PAGE and immunoblotting of residual antigenicity in hydrolysed protein formulas. *Clin Exp Allergy* 1995;25:651–658.
6. Park YW. Hypo-allergenic and therapeutic significance of goat milk. *Small Rumin Res* 1994;14:151–159.
7. Robinson F. Goats milk—a suitable hypoallergenic alternative. *Br Food J* 2001;103:198–208.
8. Bellioni-Businco B, Paganelli R, Lucenti P, et al. Allergenicity of goat's milk in children with cow's milk allergy. *J Allergy Clin Immunol* 1999;103:1191–1194.
9. Restani P, Gaiaschi A, Plebani A, et al. Cross-reactivity between milk proteins from different animal species. *Clin Exp Allergy* 1999;29:997–1004.
10. Fiocchi A, Restani P, Riva E, et al. Meat allergy: I—Specific IgE to BSA and OSA in atopic, beef sensitive children. *J Am Coll Nutr* 1995;14:239–244.
11. Fiocchi A, Restani P, Riva E, et al. Meat allergy: II—Effects of food processing and enzymatic digestion on the allergenicity of bovine and ovine meats. *J Am Coll Nutr* 1995;14:245–250.
12. Restani P, Fiocchi A, Beretta B, et al. Effects of structure modifications on IgE binding properties of serum albumins. *Int Arch Allergy Immunol* 1998;117:113–119.
13. Restani P, Fiocchi A, Beretta B, et al. Meat allergy: III—Proteins involved and cross-reactivity between different animal species. *J Am Coll Nutr* 1997;16:383–389.
14. Zeiger RS, Sampson HA, Bock SA, et al. Soy allergy in infants and children with IgE-associated cow's milk allergy. *J Pediatr* 1999;134:614–622.
15. Businco L, Cantani A, Longhi MA, Giampietro PG. Anaphylactic reactions to a cow's milk whey protein hydrolysate (Alfa-Ré, Nestlé) in infant with cow's milk allergy. *Ann Allergy* 1989;62:333–335.
16. Saylor JD, Bahna SL. Anaphylaxis to casein hydrolysate formula. *J Pediatr* 1991;118:71–74.
17. Beretta B, Conti A, Fiocchi A, et al. Antigenic determinants of bovine serum albumin. *Int Arch Allergy Immunol* 2001;126:188–195.
18. ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics. <http://www.expasy.ch/sim-prot.html>.

Requests for reprints should be addressed to:
 Prof. Patrizia Restani
 Department of Pharmacological Sciences
 Via Balzaretti, 9
 20133 Milano, Italy
 E-mail: patrizia.restani@unimi.it