

Cross-reactivity between milk proteins from different animal species

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Summary

Background Cow's milk allergy is quite frequent in the first years of human life. When breast-feeding is not possible, a cow's milk substitute must be provided for allergic subjects. Different alternatives to cow's milk have been suggested as protein sources (soy, hydrolysed proteins, goat's milk, etc.), but all these dietetic solutions are not without risks for polyallergic or more sensitive subjects.

Objective To obtain new information on the suitability of other mammalian milks for allergic children, we evaluated the cross-reactivity between milk proteins from different animal species.

Methods Milk samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). To detect antibody–antigen complexes, immunoblotting was performed by using sera from children allergic to cow's and ewe's milk (RAST class ≥ 4) and monoclonal antibodies (MoAb) specific for bovine proteins (caseins and β -lactoglobulin).

Results IgEs from children allergic to cow's milk are capable of recognizing most part of milk proteins from mammals bred in European countries (ewe, goat, buffalo), while no serum used in this study contains IgEs reacting with camel's milk proteins. Camel's milk was also not recognized from circulating IgEs from a child specifically allergic to ewe's milk. Specific antiovine monoclonal antibodies cross-reacted with proteins from other mammalian species, apart from those of camel.

Conclusions Homologies in amino acidic composition could justify the cross-reactivity observed between proteins from different animal species. On the other hand, the phylogenetic difference could be responsible for the failed recognition of camel's proteins by circulating IgEs and monoclonal antibodies.

Keywords: camel's milk, caseins, mammalian proteins, milk allergy, whey proteins

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Introduction

Cow's milk allergy is quite common in the first years of life, although there is no agreement about its actual incidence [1–5]. Bovine milk contains several proteins; they can be generally classified in two major groups: caseins and whey proteins. Because of the absence of β -lactoglobulin in human milk, this protein has been considered the most

important cow's milk allergen for a long time [6–8]. More recently, several independent studies have demonstrated that the casein fraction also has an important antigenic potential [9–11]. Moreover, while antigenicity of β -lactoglobulin can be partially eliminated by technological treatments [12], caseins maintain the capability of binding IgEs even after strong denaturing processes [10,13].

For children sensitized to cow's milk proteins whose mothers cannot breast-feed their babies, a milk substitute must be provided. Available substitutes include heat-treated

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cow's milk, goat's milk, soy milk, hydrolysed cow's milk proteins and elemental diet. At present, the most usual alternatives are soy and milk protein hydrolysed formulas but there is evidence that 10–20% of children with cow's milk allergy do not tolerate soy derivatives [14–17] and some cases of anaphylactic reaction to extensively hydrolysed formulas have been described [18–20]. On these bases, the identification of a more suitable protein source for cow's milk-allergic children represents an important goal for paediatricians and nutritionists.

Although its use is rare, goat's milk can be introduced in the diet of allergic children; this is because some children allergic to cow's milk can tolerate goat derivatives [21,22]. However, there is evidence of cross-reactivity between cow's milk and other mammalian milk proteins (goat's, sheep's and buffalo) [23–25].

To obtain new information on the suitability of other mammalian milks for allergic children, we evaluated the cross-reactivity between milk proteins from different animal species.

Materials and methods

Subjects

Children allergic to cow's milk

Serum samples were taken from six children, four males and two females; their clinical characteristics are reported in Table 1. All the children were hospitalized after instantaneous reaction to cow's milk ingestion (mainly asthma); all presented skin prick test (SPT) and radioallergosorbent test (RAST) positive responses to diagnostic reagents for cow's milk and purified milk proteins. These subjects never ingested milk or milk derivatives from other mammalian species (apart from their mother's milk). Informed consent was obtained from the parents of all children participating in this study.

Child allergic to ewe's milk

A boy, with a history of atopic dermatitis from birth in 1981, was admitted to hospital in 1990 with asthmatic symptoms as well as urticaria and rhinitis immediately after eating mozzarella cheese containing undeclared ewe's milk. Allergen extracts (Bayer, Italy) did not induce positivity at SPT with cow's whey proteins and caseins. SPT with fresh ewe's milk produced a weal larger than 6 mm in diameter (a diameter of 3 mm was considered positive). A double-blind placebo-controlled food challenge (DBPCFC) with ewe's milk was positive while open oral challenge with cow's milk was negative.

Preparation of anticasein monoclonal antibodies

Anti-casein monoclonal antibodies were prepared according to the method of Galfré *et al.* [26], with some modifications. Four to six-week-old female Balb/c mice were injected intraperitoneally with 50 µg of total casein solution (a mixture of α , β and κ caseins; Sigma Chemical Co., St Louis, MO, USA) or β -lactoglobulin (Sigma), mixed with Freund's complete adjuvant and then with two boosters, at 14-day intervals. An antigen booster was administered 3 days before removal of the spleen; the spleen cells were then hybridized with Ag8.X63 myeloma cells in the presence of 50% (w:w) polyethylene glycol, MW 1000. The fusion mixture was then diluted to 100 mL with hypoxanthine, aminopterin, thymidine (HAT) medium and distributed on feeder layers of normal Balb/c mouse peritoneal exudate cells in a 24-well plate. Wells showing anticasein or anti- β -lactoglobulin activity in their supernatant were cloned by limiting dilution. The cloned cells ($5\text{--}19 \times 10^5$ cells) were injected in Balb/c mice 1 week after pretreatment with 0.5 mL of pristane. The monoclonal antibodies harvested from the peritoneal fluid were then assayed and selected for the present study.

Name	Sex	Birth year	Cow's milk		Caseins		Whey proteins	
			SPT	RAST	SPT	RAST	SPT	RAST
A.C.	M	1990	IV	4	III	3	IV	4
B.D.	M	1989	IV	5	VI	6	–	1
B.A.	M	1991	III	6	II	6	II	3
C.M.	M	1992	IV	6	V	6	I	2
P.C.	F	1993	V	5	V	6	III	3
P.L.	F	1989	IV	4	IV	4	IV	4

SPT and RAST data are expressed as classes.

Table 1. Clinical data of children allergic to cow's milk included in the study

Milk samples

Pasteurized buffalo's, cow's, goat's and ewe's milk were kindly supplied by cheese makers from the Brescia area. Pasteurized camel's milk was kindly supplied by Dr Roberta Lodi, CNR – Milk Center, Milan. Pasteurized human milk (used to verify the specificity of reaction in immunoblotting) was from a healthy volunteer donor. She eliminated milk derivatives from the diet since her baby showed severe symptoms such as vomiting, colics and eczema. Milk samples were conserved at -30°C .

The milk samples were diluted with distilled water 1:4 (v:v); the obtained samples were treated with sample buffer (containing 0.25 mol/L TRIS-HCl pH 6.8, 7.5% glycerol, 2% SDS, 5% β -mercaptoethanol) in the ratio 1:1 (v:v) and heated in boiling water for 10 min at 100°C . An amount of sample corresponding to 1 μg protein/band was loaded onto the gel.

Purified milk proteins

Purified bovine milk proteins were purchased from Sigma Chemical Company at the highest available purification level and with the following chemical characteristics: α -casein (lyophilized powder, chromatographically purified; $\approx 85\%$ by PAGE); β -casein (lyophilized powder, minimum 90% by electrophoresis); k-casein (lyophilized powder, minimum 80% by electrophoresis); α -lactalbumin (lyophilized powder; $\approx 85\%$ by electrophoresis); β -lactoglobulin (3 $\times$ crystallized and lyophilized); bovine serum albumin (BSA; essentially globulin free; lyophilized powder; $\approx 99\%$ by agarose electrophoresis). Chemically enriched $\beta + \gamma$ caseins were prepared by our laboratory from a total casein extract [27], following the fractionated precipitation method of Tripathi and Gehrke [28].

All proteins were suspended in sample buffer at a final concentration of 1 mg/mL.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic runs were performed on a gradient (9–19%) polyacrylamide gel as previously described [10]. Gels were stained according to the method of Neuhoff *et al.* [29].

Immunoblotting

After SDS-PAGE, transfer of proteins from the gel onto the polyvinylidene difluoride membrane (PVDF, Immobilon P; Millipore, Bedford, MA, USA) is achieved by electrophoretic elution (wet transfer). The transfer buffer was: 25 mmol/L TRIS, 193 mmol/L glycine and 20% methanol. To verify the protein transfer and the precise protein position, a solution of

0.2% Ponceau S (in 3% TCA and 3% sulfosalicylic acid) was used. After the destaining, PVDF membranes were blocked with 1% gelatine and washed three times with 0.25% gelatine (BioRad) solution (in 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L TRIS, 0.05% TRITON-X) to prevent non-specific adsorption of the immunological reagents. Membrane was then immersed in 10 mL of 0.25% gelatine solution, containing 300 μL of serum from allergic children or 10 μL of monoclonal antibodies. Antigen–IgE complex was detected using goat antihuman IgE antibodies (Sigma) while antigen–IgG complex was detected using goat antimouse IgG antibodies (Sigma), both labelled with alkaline phosphatase. Finally after incubation in the bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution, an intense black–purple precipitate developed at the site of enzyme binding. When unspecified, chemicals were from Merck (Darmstadt, Germany). Quantitative analyses were performed using a gel scanner (Sharp JX-330, Pharmacia Biotech, Sweden) and the Image MasterTM 1D Software. It allows the quantification of proteins calculating the average density of pixels across the band length and integrating over the band width. Grades of positive reactions were determined by comparing the precipitate colour on membrane with a self-prepared scale of black–purple.

Since sera from nonallergic subjects sometimes showed responses of grade I, only responses $\geq$ grade III were considered specific reactions.

Results

In Fig. 1, the protein patterns of milk from the mammalian species included in this study are reported. In cow's milk,

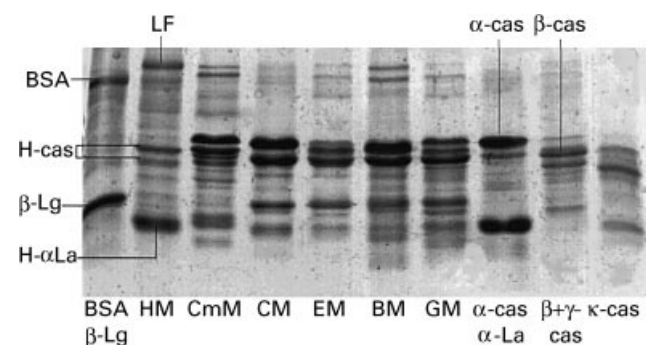


Fig. 1. SDS-PAGE of purified cow's milk proteins and milk samples from different animal species. α -La = cow's α -lactalbumin; H- α -La = human α -lactalbumin; β -Lg = cow's β -lactoglobulin; BSA = bovine serum albumin; cas = casein, H-cas = human casein; LF = lactoferrin. HM = human milk; CmM = camel's milk; CM = cow's milk; EM = ewe's milk; BM = buffalo's milk; GM = goat's milk.

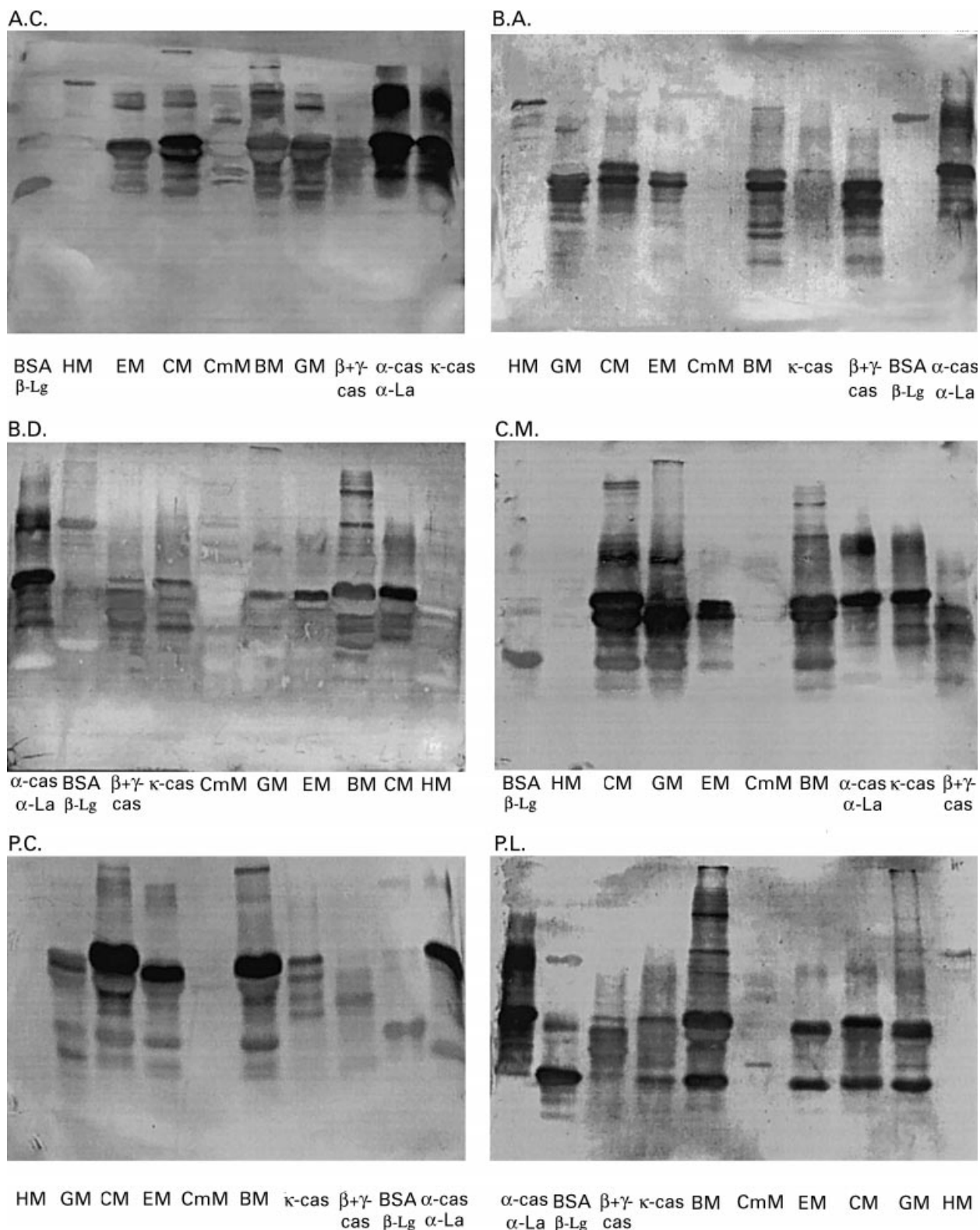


Fig. 2. IgE-antigen complexes obtained in immunoblotting with the sera of 6 children allergic to cow's milk α -La = cow's α -lactalbumin; β -Lg = cow's β -lactoglobulin; BSA = bovine serum albumin; cas = casein; HM = human milk; CmM = camel's milk; CM = cow's milk; EM = ewe's milk; BM = buffalo's milk; GM = goat's milk

Table 2. Positive responses to purified cow's milk proteins observed in immunoblotting

Name	α -casein	$\beta + \gamma$ caseins	k-casein	α -lactalbumin	β -lactoglobulin	BSA
A.C.	V	II	IV	—	II	II
B.D.	V	V	III	—	—	II
B.A.	V	II	II	—	—	II
C.M.	V	IV	—	—	III	I
P.C.	V	II	II	III	III	I
P.L.	V	III	II	—	IV	I

Data are expressed as 'grades' according to our self-prepared scale of colour (see Materials and methods). Only grades $\geq$ III are considered specific reactions.

the four major bands correspond to α -casein, β -casein, β -lactoglobulin and α -lactalbumin (from top to bottom). Buffalo's milk shows a similar pattern although there is a 5th band at the bottom. Compared with those of cow's milk, ewe's and goat's milk present a lower amount of α -casein while the β -fraction is the most abundant; a third component between α - and β -caseins is also visible. Camel's milk shows a quite different pattern with a higher number of protein bands in the area of caseins and the absence (or traces) of β -lactoglobulin. Human milk has a more complex protein pattern; in fact, apart from caseins (β and k fractions) and α -lactalbumin, it presents a band with high MW corresponding to lactoferrin.

In Fig. 2, the positive responses obtained in immunoblotting with the sera of children allergic to cow's milk are shown. The presence in the gel of purified cow's milk proteins permits us to better classify the allergic profiles of each subject. The positive responses to purified cow's milk proteins are summarized in Table 2. All the children included in this study presented a high quantity of circulating IgEs specific for α -casein (class V); different patterns of reactivity were observed for $\beta + \gamma$ and k-caseins. Since γ caseins (γ 1,2 and 3) are known fragments of β -casein [30], we considered these proteins together. The high specificity of IgEs for α -casein permits to identify traces of this protein in other purified fractions; this fact is evident in k-casein pattern.

We observed only one and three significant responses (grade $\geq$ III) against α -lactalbumin and β -lactoglobulin, respectively. These latter data are not always in agreement with the SPT/RAST results (Table 1) but, as described previously, whey proteins are labile antigens so that denaturation with SDS and β -ME can reduce their capability of binding IgEs.

Considering the immunoblotting obtained after incubation of the sera with milk from different animal species, it is possible to show that:

- for every child, the reactions to purified cow's proteins are confirmed by those observed in cow's milk samples;

- circulating IgEs of all children recognized buffalo's and ewe's milk proteins. In particular, the reactivity is evident for α -casein (all subjects) and β -lactoglobulin (C.M., P.C. and P.L.);
- compared with cow's milk, the reaction to goat's milk proteins is comparable for three children (B.A., C.M., P.L.) and lower for the other three subjects (A.C., B.D., P.C.);
- no child showed positive response to camel's milk proteins.

No significant IgE-antigen complex was observed with proteins of human milk, which was included in the gels as a negative control; this confirmed the specificity of the immunoreactions.

Figure 3 shows the immunoblotting obtained after incubation with the serum of the child allergic to ewe's milk. Circulating IgEs recognize only ewe's and goat's caseins, while no reactivity is present vs cow's and buffalo's

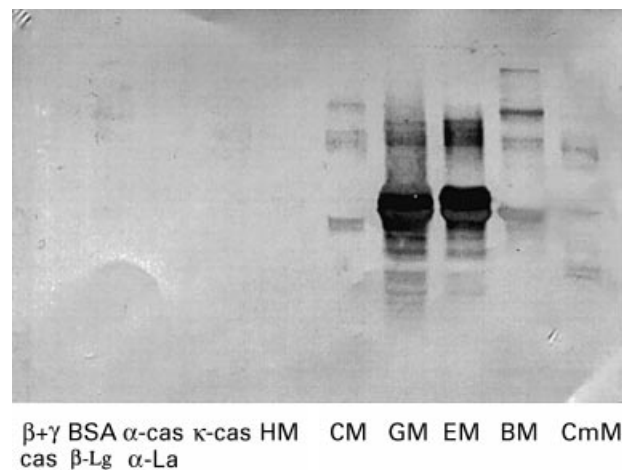


Fig. 3. IgE-antigen complexes obtained in immunoblotting with the serum of a child allergic to ewe's milk. Abbreviation as in Figs 1 and 2.

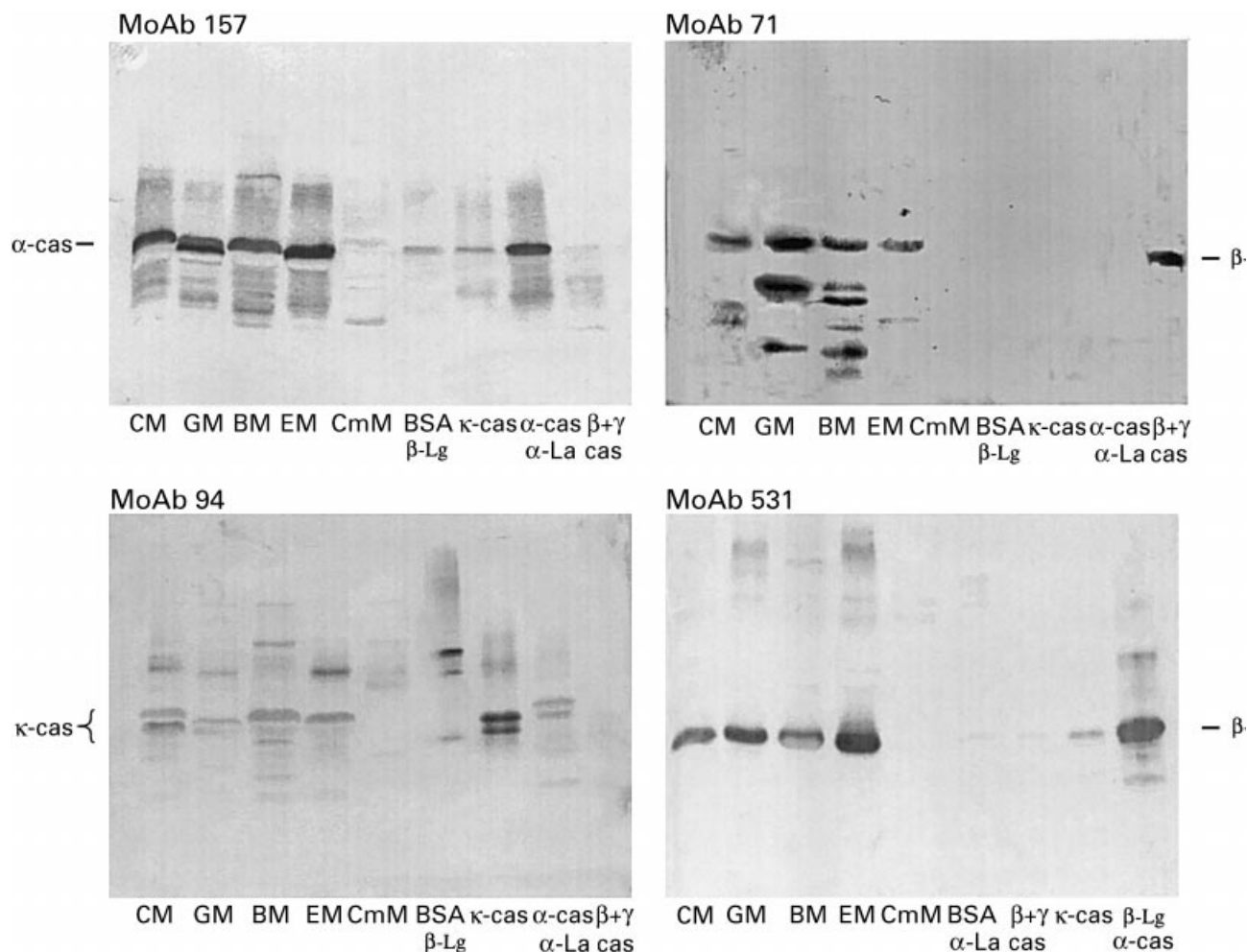


Fig. 4. IgG-antigen complexes obtained in immunoblotting with the monoclonal antibodies specific for cow's milk proteins. Monoclonal antibodies: MoAb 157 = anti α -casein, MoAb 71 = anti β -casein, MoAb 94 = anti κ -casein and MoAb 531 = anti β -lactoglobulin. Abbreviation as in Figs 1 and 2.

milk. Also in this case, camel's milk proteins are not recognized by circulating IgEs.

Figure 4 shows the immunoblottings obtained with monoclonal antibodies (MoAb); MoAb 157 recognizes α -casein, MoAb 71 β -casein, MoAb 94 κ -casein and MoAb 531 β -lactoglobulin. Cross-reactivity between cow's proteins and other mammalian species (ewe, goat and buffalo) is evident both for caseins and for β -lactoglobulin. Only camel's proteins are never recognized by monoclonal antibodies; in particular, the negative response obtained with MoAb 531 can also be associated with the absence of β -lactoglobulin in camel's milk.

Discussion

The best alternative diet for children with allergy to cow's milk proteins remains to be defined. The important

differences between human and cow's milk both for the qualitative and quantitative composition suggest that new alternative protein sources could be considered for the production of hypoallergenic formulas.

Since goat's milk can sometimes be tolerated by children sensitized to cow's milk [21,22], we decided to verify the reactivity of circulating IgEs from allergic children (RAST class ≥ 4) to milk proteins from the mammalian species usually bred in our country. As an 'exotic' protein source we studied camel's milk.

Considering the protein pattern as identified by SDS-PAGE, we can observe that cow's and buffalo's milk present a similar protein composition; ewe's and goat's milk are similar to cow's milk but a reduction of α -casein fraction is evident. Camel's milk shows a different pattern with numerous bands in the area of caseins and the absence of β -lactoglobulin.

When the sera of six children highly allergic to cow's milk were used in the immunoblotting technique, it was possible to show the formation of IgE-antigen complexes with all milk tested apart from camel's milk. Compared with cow's milk, three out of six sera showed a lower reaction to goat's milk. This result could be justified by the lower amount of α -casein and could explain why some allergic children are able to tolerate goat's milk, as reported previously [21,22].

In the case of the child specifically allergic to ewe's milk, we found reactivity only to ewe's and goat's caseins. Since no reactivity was observed vs cow's milk (normally consumed by this subject), we must conclude that the epitope or the epitopes responsible for the sensitization are different from those involved in cow's milk allergy.

The IgE binding to milk proteins from different animal species, obtained with the sera of cow's milk-allergic children, must be considered as a cross-reactivity for different reasons:

- these subjects never ingested milk or milk derivatives from mammalian species other than cow (apart in some cases from the mother's milk);
- when sera from allergic children were pre-incubated during the immunoblotting procedure with cow's milk before the addition to the incubation chamber, IgE-antigen complexes on membrane were modified: all reactions disappeared apart from those with cow's milk proteins, which were only reduced (data not shown);
- finally, definitive results can be obtained by the use of specific (anticow) monoclonal antibodies. In fact, monoclonal antibodies produced specifically against cow's proteins recognized those from some mammalian species: ewe, goat, buffalo. This phenomenon can be justified by the sequence homology and by the presence of identical or very similar epitopic areas.

The absence of reactivity observed with monoclonal antibodies demonstrates that cow's and camel's proteins do not contain homologous epitopic areas.

Some conclusions can be drawn from the results of this research:

- the use of goat's milk (a sporadic clinical practice) could be suggested only in well-selected cases and after a suitable modification from the nutritional point of view. Its use is not free of risk: 50% of our subjects had circulating IgEs capable of recognizing goat's proteins with the same affinity shown for cow's milk. Moreover, no subject showed a negative pattern of response in immunoblotting;
- camel's milk might be a promising new protein source for allergic children and it might be used in modified formulas.

From the nutritional point of view, camel's milk is an interesting protein source; its amino acid composition is

similar to that of cow's milk as shown previously [31]. The absence of proteins having electrophoretic mobility comparable with β -lactoglobulin seems to be important for two major reasons: (a) there is analogy with human milk, and (b) one of the most important cow's milk allergens could be lacking.

The availability of camel's milk on the international market is not a problem; in fact, according to FAO statistics there are at least 17 million camels in the world mainly in Africa (Somalia, Ethiopia, Mauretania, Kenya) and Asia (mainly India, China, Pakistan) [32].

Although the use of camel's milk needs clinical studies able to verify its suitability for the human diet and in particular for allergic children, we think that this alternative protein source might be successfully used in modified formulas. Technological processes, such as heat treatment or hydrolysis, could be set up to reduce the possibility of new sensitization.

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