Allergenicity of mare's milk in children with cow's milk allergy

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Background: Cow's milk allergy is a common disease of infancy and early childhood. If the baby is not breast-fed, a substitute for cow's milk formula is necessary.
Objective: The aim of this study was to investigate, in vitro and in vivo, the allergenicity of mare's milk in a population of selected children with severe IgE-mediated cow's milk allergy.
Methods: Twenty-five children (17 male and 8 female) aged 19 to 72 months (median age 34 months) with IgE-mediated cow's milk allergy were selected for this study. All the children underwent skin prick tests with cow's milk and mare's milk and double-blind placebo-controlled oral food challenge (DBPCOFC) with fresh cow's milk, fresh mare's milk, and, as placebo, a soy formula (Isomil, Abbott, Campoverde, Italy). We performed immunoblotting of cow's and mare's milk developed with IgE from allergic children.
Results: All the children showed strong positive skin test responses to cow's milk (4+); 2 children had positive skin test responses to mare's milk (2+). All children had positive DBPCOFCs to cow's milk; one child had a positive DBPCOFC to mare's milk. No children reacted to the placebo (Isomil). In the cow's milk, some proteins are able to strongly react with human IgE; when the sera are tested with mare's milk, the bands corresponding to the same proteins are recognized by a lower percentage of sera.
Conclusion: These data suggest that mare's milk can be regarded as a good substitute of cow's milk in most children with severe IgE-mediated cow's milk allergy. It would be prudent, however, to confirm its tolerability by a supervised titrated oral challenge test. (J Allergy Clin Immunol 2000;105:1031-4.)

Key words: *Cow's milk allergy, cow's milk substitute, mare's milk, mare's milk allergy*

Cow's milk allergy (CMA) is a common disease of infancy and early childhood, with a prevalence of approximately 2.5% during the first 3 years of life.[1] If the baby is not breast-fed, a substitute for cow's milk (CM) formula to feed babies with CMA is necessary because in early life milk is the only source of nutrition. At the moment, the substitutes are soy formula and casein or whey extensively hydrolyzed formula (eHF).[2] The drawbacks of these products are potential allergenicity, unpleasant taste (eHF), high cost (eHF), and nutritional deficiencies (eHF).[3-6] Goat's milk, which is prescribed by some physicians as a CM substitute, has induced allergic reactions in most children with CMA.[7] Interestingly, ass milk was successfully used in children with multiple food allergy, including CMA.[8]

Abbreviations used

CM:	Cow's milk
CMA:	Cow's milk allergy
DBPCOFC:	Double-blind, placebo-controlled oral food challenge
eHF:	Extensively hydrolyzed formula
MM:	Mare's milk
MW:	Molecular weight
PBS-T:	PBS + Tween 0.05% vol/vol
SDS-PAGE:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPT:	Skin prick test

The aim of this study was to investigate, in vitro and in vivo, the allergenicity of mare's milk (MM) in a population of selected children with severe IgE-mediated CMA. To our knowledge no such studies have been done before.

MATERIAL AND METHODS

Patients

Twenty-five children (17 male and 8 female), aged 19 to 72 months (median age 34 months) with CMA, were selected for this study. The diagnosis of CMA was made on the basis of personal history and physical examination and was confirmed by positive responses both to skin prick test (SPT) to CM and to double-blind, placebo-controlled oral food challenge (DBPCOFC).

The symptoms reported by the children after the ingestion of CM were atopic dermatitis (19), atopic dermatitis and asthma (4), asthma (1), and urticaria (1).

All the children underwent SPT with CM and MM and DBPCOFC with fresh CM, fresh MM, and, as placebo, a soy formula (Isomil, Abbott, Campoverde, Italy).

Sera were collected from all 25 children to be used in the immunoblotting test. Three of the 25 sera were selected for the immunoblotting inhibition experiments on the basis of their clinical and serologic reactivity (No. 13: positive in SPT for both CM and MM, challenge test positive for MM, positive in blotting for both CM and MM; No. 14: positive in SPT for CM and negative for MM, challenge test negative for MM, positive in blotting for both CM

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and MM; No. 24: positive in SPT for CM and negative for MM, challenge test negative for MM, positive in blotting for CM and negative for MM).

SPTs

Skin testing was done by the prick method on the volar surface of the forearm. The prick tests were read after 20 minutes and considered positive when the wheal was 3 mm greater than the negative control. Children were tested with isotonic saline solution as a negative control, histamine (10 mg/mL) as a positive control (SARM, Rome, Italy), and undiluted pasteurized fresh CM and MM.

DBPCOFC

Challenge tests were performed in a day-hospital setting, administering fresh CM or MM or, as placebo, a soy formula (Isomil) as follows: a drop was put on the inner side of the lower lip, 5 mL was ingested after 5 minutes and 25 mL after 30 minutes. If no symptoms appeared, 100 mL was given after 30 minutes. After the last administration of the tested milk, the children were kept under observation for at least 4 hours and then discharged. The next challenge test was done 1 week later.

SDS-PAGE and electrotransfer

Protein content of the milk samples was assayed according to the method of Bradford.[9]

SDS-PAGE and electrotransfer were carried out essentially as previously described[10] in a minielectrophoresis and blotting system (Bio-Rad, Richmond, Calif). The electrophoresis was run under constant current (15 mA in the stacking gel and 30 mA in the separating gel). CM and MM (10 μg/mL protein/well) were reduced by 5% vol/vol 2-mercaptoethanol, treated at 100°C for 5 minutes, spun, and then applied to the 15% wt/vol polyacrylamide gel. The gel was stained with 0.05% Coomassie brilliant blue (Imperial Chemical Industries, Macclesfield, UK) in water/methanol/acetic acid (50:40:10). The separated proteins were then tranferred from the SDS gel to a nitrocellulose membrane overnight under constant amperage (80 mA).

Immunoblotting and immunoblotting inhibition

The blotted nitrocellulose strips were blocked l hour with 3% wt/vol gelatin (Sigma, Milan, Italy) in PBS, pH 7.2. After being washed in PBS + Tween 0.05% vol/vol (PBS-T), strips were incubated overnight with 1 mL per strip of individual sera from the 25 allergic children and from 1 healthy subject as negative control diluted 1:5 in PBS-T at room temperature. Strips were washed again and then incubated overnight with 1 mL per strip (about 30,000 counts/min/strip) of iodine 125–1abeled goat antihuman IgE (Bioallergy, Rome, Italy) diluted in PBS-T. After being washed in PBS-T, the reactive bands were detected by autoradiography at –80°C for 4 days with the use of x-ray film (Kodak Diagnostic Film X-Omat AR, Eastman Kodak Company, Rochester, NY).

Immunoblotting inhibition experiments were performed essentially as described by Barletta et al.[10] IgE from 3 of the 25 allergic patients was diluted 1:5 or 1:10 in PBS-T and preincubated overnight at room temperature with 50, 10, or l μg of protein per milliliter of CM or MM. The blotted strips were incubated overnight at room temperature with the mixtures and then overnight with ^{125}I-1abeled goat antihuman IgE. Developing was carried out as described for direct immunoblotting. The results of inhibition experiments were quantitatively analyzed by densitometric scanning of the autoradiographed films on a Multi-Analist/PC-Densitometer (Bio-Rad). Percentage of inhibition was calculated on the peak's area corresponding to each component exhibited by the densitometric analysis.

RESULTS

Skin tests and DBPCOFC

The results of SPT and DBPCOFC to CM and MM are reported in Table I. All the children showed strong positive skin test responses to CM (mean diameters: wheal = 10 mm, erythema = 16 mm); 2 children had positive skin test responses to MM (wheal 3 and 2 mm, erythema 20 and 4 mm). All children had positive DBPCOFC to CM, 1 child (serum No. 13) had positive DBPCOFC to MM. This child also had positive SPT to MM and had urticaria after the administration of 60 mL of MM. In the CM challenge test the main symptoms were urticaria in 17 children, rhinitis and/or wheezing in 3, vomiting in 3, and angioedema in 2. All the positive responses to the challenge occurred within 1 hour (range 2 minutes to 1 hour). The median dose of CM that gave a positive response to the challenge test was 20 mL (range 1-100 mL). No children reacted to the placebo (Isomil).

Immunoblotting and immunoblotting inhibition

SDS-PAGE analysis in reducing condition of the whole CM and MM (Fig 1, lanes *A* and *B*, respectively) showed the presence of many components displaying a molecular weight (MW) ranging from 14 kd to about 80 kd. The separated components detected in the CM corresponded to the milk proteins identified as relevant allergens in both whey and curd fractions. In fact, α-lactalbumin (14.2 kd), β-lactoglobulin (18.3 kd), and BSA (66.3 kd), together with the group of caseins (MW from 23 to 36), could be identified. Caseins could be considered as major components in terms of band intensity. Moreover, because β-lactoglobulin occurs naturally in the form of a 36-kd dimer, the corresponding band probably comigrates with the caseins.

The electrophoretic profiles of CM and MM showed similar patterns, with a component of about 23 kd, probably belonging to the casein group that, together with α-lactalbumin (14.2 kd), could be regarded as the most intense component.

The immunoblotting of CM and MM developed with IgE from allergic children is reported in Fig 2 (*A* and *B*, respectively). In the CM, some proteins (corresponding to α-lactalbumin, β-lactoglobulin, and caseins) strongly reacted with human IgE. In terms of frequency, α-lactalbumin and β-lactoglobulin were recognized by 18 of 25 sera (72%) and 17 of 25 (68%), respectively. The casein group together with the 36-kd dimer was recognized with variable intensity by 18 of 25 sera (72%); the bands at 66 kd (BSA) and 80 kd (lactoferrin) were recognized, respectively, by 18 of 25 sera (72%) and by 7 of 25 allergic children (28%). When the sera were tested with MM, the bands corresponding to the same proteins were recognized by a much lower percentage of sera. Six of 25 sera (24%) reacted with α-lactalbumin and β-lactoglobulin, whereas overall caseins were recognized by 5 of 25 sera (20%). On the other hand, the band at 80 kd was weakly recognized by 8% of sera. No sera were able to

detect any component in the 45- to 66-kd MW regions. The normal serum used as negative control did not recognize any band in either CM or MM.

To investigate whether IgE from allergic children recognized epitopes on CM shared by MM, we set up inhibition experiments, using CM as the blotted antigen. Results obtained by densitometric analysis of blotting inhibition are shown in Table II. CM was able to cause a high level of IgE inhibition with all the sera tested, ranging from 100% to 62% for the different components analyzed. On the contrary, when MM was used as inhibitor, the IgE reactivity against the majority of the CM components was only poorly inhibited even at the highest amount of inhibitor. When an inhibition could be recorded, it never reached 50% of IgE inhibition, the highest inhibition value being 28% with lactoferrin at 50 μg/mL of inibitor.

DISCUSSION

This is the first study that investigates the in vitro and in vivo allergenicity of MM in children with proved CMA.

The results of this study indicate that MM is tolerated by 96% of the children with CMA. Only 1 of the 25 children had a positive challenge test to MM. These data are interesting because the enrolled subjects formed a highly sensitized selected group of children, as shown by the following data. The median age of the children was 34 months, and the majority of the children were more than 3 years old. It is well known that CMA usually disappears within the first 3 years of life, and only extremely sensitized children continue to be allergic to CM after 3 years of age. The positive response in SPT to CM was 4+ in all the children, and a very minute amount of CM was required to trigger a positive response to the challenge.

It has been shown that the amino acid sequences of some MM proteins differ from those of CM proteins.[11] Three different kinds of α-lactalbumin, designated A, B, and C, have been isolated in MM. Comparison of the sequences of B and C with that of A indicates 3 and 4 amino acid exchanges, respectively. The primary structures of equine α-lactalbumin B and C have been determined. The phylogenetic difference of equine α-lactalbumin B/C from bovine α-lactalbumin B is indicated by 39 and 40 amino acid exchanges, respectively.[11] These differences in the amino acid sequences of bovine and equine proteins could account for the different levels of inhibition obtained with the 2 milks tested against CM as antigen in immunoblotting. In fact, the epitopes relevant for IgE binding to CM could be different or even lacking completely in MM.

Ass' milk was successfully used in children with CMA.[8] Iacono et al showed that ass' milk was tolerated without any problems in 9 infants with severe symptoms (vomiting, diarrhea, failure to thrive, shock) resulting from CMA. Ass and mare have the same phylogenetic origin (Equidae), which differs from that of cow (Bovidae).

The composition of MM is much more similar to human milk than is CM.[11,12] The protein content is low (1.3 to 2.8 g/100 mL), and it does not produce an excessive renal load of solute; the protein fraction is particularly rich in whey proteins (35%-50%). The high lactose content (5.8-7.0 g/100 mL) makes it pleasant to eat and also qualitatively preferable to a semielemental formula containing protein hydrolysates or soy formulas that contain carbohydrates other than lactose. It is known that

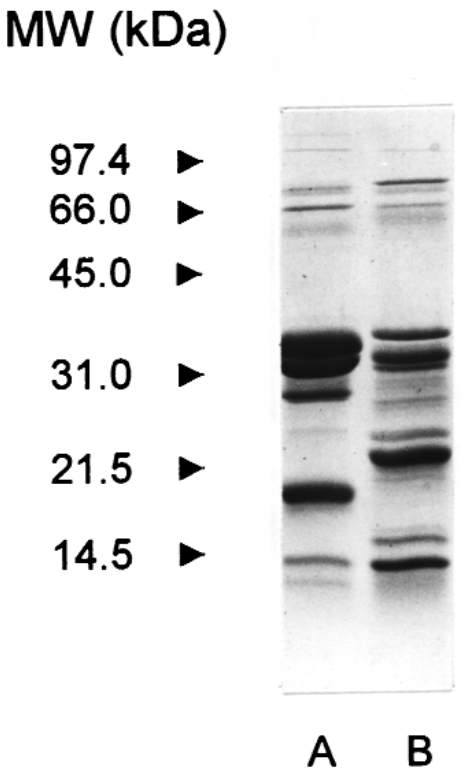

FIG 1. CM *(lane A)* and MM *(lane B)* after separation by SDS-PAGE under reducing conditions and Coomassie brilliant blue staining.

TABLE I. Skin test and challenge test responses to CM and MM in 25 children with CMA

	CM		MM	
	No.	%	No.	%
Positive skin tests	25/25	100	2/25	8
Positive DBPCOFC with 22.8 mL (mean) of CM (range 1-100 mL)	25/25	100	1/25	4

TABLE II. Densitometric analysis of blotting inhibition

	Serum No. 13		Serum No. 14		Serum No. 24	
Protein	CM*	MM†	CM*	MM†	CM*	MM†
α-Lactalbumin	ND	ND	100%	0%	100%	14%
β-Lactoglobulin	ND	ND	81%	0%	100%	15%
Caseins	63%	21%	83%	14%	66%	0%
BSA	ND	ND	100%	3%	100%	9%
Lactoferrin	ND	ND	ND	ND	100%	28%

Maximum percent of inhibition (obtained with the highest inhibitor concentration) is reported. *ND,* Protein bands not detectable by the densitometer.
*CM as inhibitor.
†MM as inhibitor.

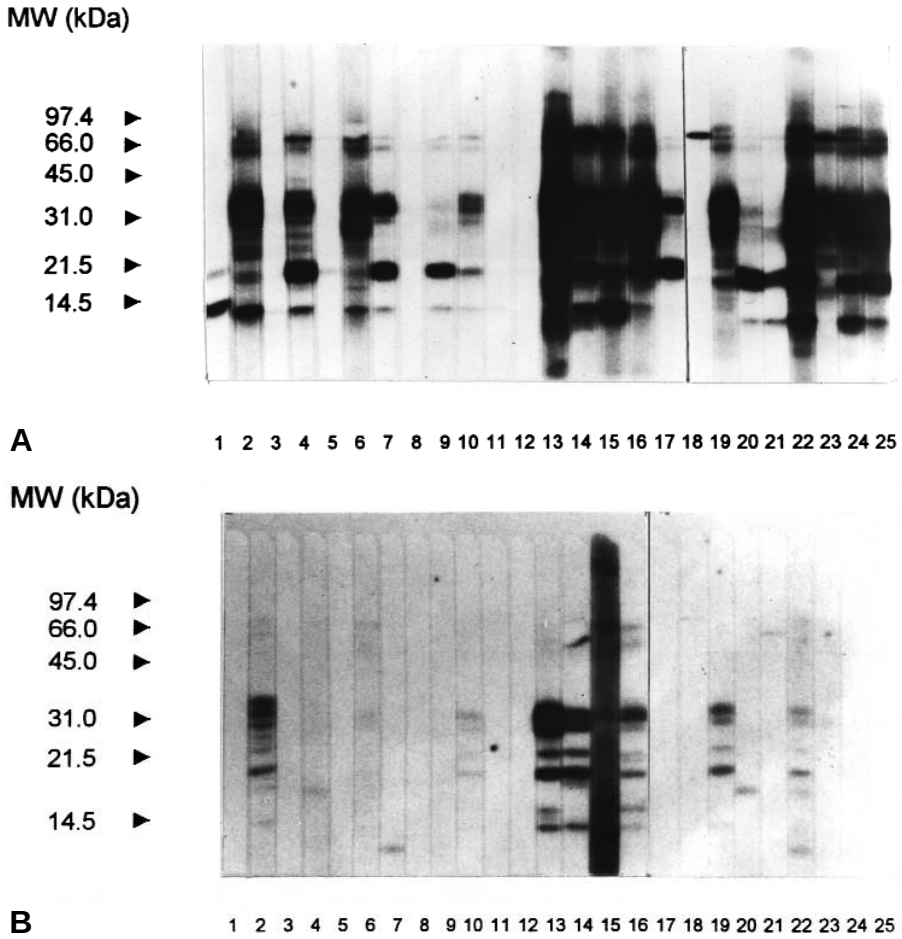

FIG 2. CM **(A)** and MM **(B)** immunoblotting developed with individual sera from allergic children *(lanes 1 to 25).*

lactose stimulates the intestinal absorption of calcium, which can have a favorable effect on bone mineralization in the first few months after birth. The calcium/phosphorus ratio is 1.7, which is very close to the optimal value for calcium absorption and metabolism.[12,13]

The distribution of diglycerides and triglycerides in MM is very similar to that found in human milk and very different from the distribution of glycerides in CM. The ratio between unsaturated and saturated fatty acids is 1.32 (0.45 for CM) and the ratio between polyunsaturated and monounsaturated fatty acids is 0.83 (0.08 for CM).[12,13]

These data strongly suggest that MM, with appropriate modifications, can be regarded as a good substitute of CM in children with severe IgE-mediated CMA.

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