

Original article

Evaluation of the residual antigenicity and allergenicity of cow's milk substitutes by *in vitro* tests

Background: This study aimed to investigate the presence of residual allergenic cow's milk proteins (CMP) in some milk substitutes employed in the treatment of cow's milk allergy (CMA). These allergens may interfere with the treatment, and elicit allergic reactions in sensitized individuals.

Methods: The protein composition of the different extracts was evaluated by Lowry's method and tricine SDS-PAGE. Different immunoenzymatic methods were used (ELISA, EAST and immunoblotting) to quantify total serum IgE and specific serum IgE, as well as to detect the presence of antigenic and allergenic components.

Results: The results showed a higher protein content in mammalian milks (cow, sheep, mare, goat, and human) than in hydrolyzed substitutes (partially or extensively hydrolyzed casein or whey proteins). Residual native, processed, or contaminant polypeptides have been identified in the moderate hydrolysates, whereas extensive hydrolysates did not show the presence of residual components by immunoblotting. However, specific antibodies with capacity to bind to peptides have been detected by EAST and ELISA, suggesting that extensive hydrolysates contain residual peptides that preserve immunoreactive epitopes. We were unable to demonstrate either residual antigenicity or allergenicity in an amino-acid-based formula.

Conclusions: Immunoenzymatic methods were used to detect the presence of cross-reactive components in mammalian milks. Residual allergenic components from cow's milk could be identified in both the moderate and extensive hydrolysates analyzed. This information may be relevant to the treatment of CMA.

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Allergy to cow's milk (CM) is quite common in early childhood. Around 2.5% of neonates experience hypersensitivity reactions to cow's milk proteins (CMP) during the first year of life (1). Prevention and treatment of this condition are relevant in early life; these goals, however, are difficult to achieve, since most infant formulas are based on CM.

The risk of allergy is determined by interactions between genetic and environmental factors. At present, attempts to decrease environmental influence are the principal target in prevention. The development of CMA is highly associated with early exposure to CM.

Abbreviations. CM: cow's milk; CMP: cow's milk proteins; CMA: cow's milk allergy; β -Lg: β -lactoglobulin; α -La: α -lactalbumin; BSA: bovine serum albumin; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MW: molecular weight; EAST: enzyme allergosorbent test; HF: hydrolyzed formula; eHF: extensively hydrolyzed formula; pHF: partially hydrolyzed formula; OPD: σ -phenylenediamine; 5-BCIP/NBT: 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; OD: optical density.

Primary prevention is focused on sensitization, and, with regard to prenatal sensitization, there is no certain evidence of a beneficial effect of maternal diet during pregnancy (2, 3). Infancy represents the period in which the individual may be at the highest risk of sensitization. Therefore, allergy prevention after perinatal identification of such at-risk neonates would be more effective to decrease the cases of atopic diseases during infancy (4).

Immunoenzymatic methods may detect the presence of residual antigenic and allergenic – native or processed – components in a CM substitute. Along with the precise identification of the offending allergen, such findings are relevant to the initiation of the treatment.

Many CMP have been identified as allergens of CM since they can elicit IgE-mediated responses. Several studies have identified and characterized immunoreactive epitopes in caseins (5, 6), described as the main antigenic and allergenic components of CM (7–9).

There is evidence that the mode of early infant feeding influences the development of food allergy. Exclusive

breast-feeding may protect children from the development of atopic diseases (10). Since native CMP have been detected in breast milk (3, 11), avoidance of intake of the offending allergens by the mother during the first 3 months of breast-feeding may protect sensitive children against the onset of allergic symptoms. Prolonged breast-feeding and a delayed introduction of solid food may reduce the incidence of CMA (12). When breast milk is insufficient, a milk substitute must be employed. Soy and protein hydrolyzed formulas (HF) are the substitutes most widely used for allergic individuals. Most preventive studies have included the use of a formula based on hydrolyzed CMP for early infant feeding. Residual allergenic activity has been described in partially (pHF) and extensively hydrolyzed formulas (eHF) (13, 14), and it may cause anaphylactic reactions in sensitized children (15). Milks from different animal species have also been indicated for infant feeding. However, the main allergenic components are similar to those of CM, resulting in cross-reactivity and, hence, allergenicity (9).

It is therefore of clinical relevance to know the residual allergenicity of the dairy substitutes employed in the prevention and treatment of CMA. The indiscriminate labeling of infant formulas as hypoallergenic, based upon the degree of hydrolysis of CM proteins, without considering the allergenicity of the residual components and, therefore their safety in children with CMA, poses the risk of adverse reactions in allergic individuals. Since atopic diseases are multifactorial disorders, it is not possible to associate, *a priori*, the presence of allergenic residual components with the appearance of allergic reactions.

We have analyzed a variety of milk substitutes, using *in vitro* immunoenzymatic methods and specific antisera, monoclonal antibodies, and sera from CMA patients as a source of IgE antibodies.

Material and methods

Infant formulas and milk proteins

The milk products included NAN HA (Nestlé), a partially hydrolyzed whey formula; Kas 1000 (Kasdorf), a partially hydrolyzed casein formula; Pregestimil® (Mead Johnson), an extensively hydrolyzed casein formula; HUMANA HA 1 (HUMANA), a partially hydrolyzed whey and casein formula; APTAMIL HA 1 (Milupa), a partially hydrolyzed whey and casein formula; BEBA HA 1 (Nestlé), a partially hydrolyzed whey formula, with added hydrolyzed BSA; BEBA HA 2 (Nestlé), a partially hydrolyzed whey formula, with added hydrolyzed BSA; Neocate (SHS International), an amino-acid-based formula; milk from different animal species (cow, sheep, goat, and mare) supplied by different farms; and maternal milk. Purified bovine milk proteins were purchased from Sigma Chemical Co at the highest available purification level (α_s -casein, β -casein, κ -casein, β -lactoglobulin [β -Lg], and α -lactalbumin [α -La-I]). Commercial fractions of total casein (Hammersham) were also employed.

Protein extracts from powdered samples were prepared by adding phosphate-buffered saline, pH 7.4 (10 mg/ml) and incubated overnight at 4°C with continuous shaking. Lipids were extracted with

chloroform, and samples were dialysed overnight against PBS, using a membrane of 2000-Da cutoff (SPECTRA/POR MWCO 2000). Samples were stored at -20°C until used.

The total soluble protein content of preparations was measured by Lowry's method, with bovine serum albumin as the reference (16). In some cases, the protein concentration was also determined by the Microkjeldahl method.

Monoclonal and polyclonal antibodies

Casein-specific monoclonal antibodies were prepared by injecting 6-week-old female Balb/c mice intraperitoneally with 25 μ g of CMP emulsified 1:1 in complete Freund's adjuvant, followed by three boosts with 15 μ g of proteins in Freund's incomplete adjuvant at 3-week intervals. Serum-specific antibody titers were determined by indirect ELISA. Hybridomas were obtained by the Galfre-Milstein technique (17). The casein specificity of the monoclonal antibodies harvested from the peritoneal fluid was assayed by immunoblotting and indirect ELISA, employing commercial milk proteins and electroeluted casein components from CM extract. Three monoclonal antibodies were selected for the study with high specificity for α -casein (1D5-I), β -casein (2A1-I), and κ -casein (3B5-I).

CM polyclonal antiserum was prepared by inoculating NZW rabbits with 100 μ g of CMP emulsified with complete Freund's adjuvant. A series of four injections (50 μ g) of the same antigen in incomplete Freund's adjuvant was administered every 2 weeks. Antibody titers were determined by indirect ELISA.

Electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts was carried out in a 14% polyacrylamide gel with a discontinuous buffer system, according to Laemmli (18). Gels were migrated on a Bio-Rad Mini-Protein III (Bio-Rad, Richmond, CA, USA) at 100 V through the stacking gel, and at 150 V through the running gel, either with or without 5% β -mercaptoethanol. A 10- μ l sample of the protein extract was loaded onto each well. The amount of protein loaded was 4–13 μ g. Gels were fixed and stained with either Coomassie brilliant blue R250 or silver staining, according to the protein content of the sample. The relative molecular weights of the resolved protein fractions were compared with those of marker proteins.

Tricine SDS-PAGE was performed as described by Schagger et al. (19). Electrophoresis was performed initially at 30 V constant for 1 h and then at 85 V constant for 5 h.

Separated proteins were electrotransferred from the gel to 0.45-nm pore size nitrocellulose membranes (SMI) for 45 min at 300 mA (20). Membranes were stained with Ponceau S to demonstrate successful transfer of protein. Afterward, they were incubated for 1 h in a blocking buffer containing 5% horse serum in PBS, pH 7.4, and cut into 5-mm strips for immunostaining. Sera from patients and controls (diluted 1:5 in blocking buffer), rabbit polyclonal antiserum (diluted 1:500 in blocking buffer), and monoclonal antibodies were added to detect the presence of antigenic and allergenic components in each extract. Human sera were incubated for 12 h at room temperature, and monoclonal antibodies and polyclonal antiserum were incubated for 1 h at 37°C.

After thorough washing (0.05% Tween-PBS), incubation with different conjugate systems was assessed: biotinylated goat anti-human IgE (ϵ -chain specific) antibody (Vector, CA, USA) diluted 1:2000 in blocking buffer (2 h at 37°C), and then ExtrAvidin-alkaline phosphatase conjugate (Sigma), diluted 1:1000 for 1 h at 37°C, were employed to reveal human serum IgE antibodies; antimouse IgG (whole molecule) horseradish peroxidase (HRP) conjugate (Bio-Rad), diluted 1:2000 and incubated for 1 h at 37°C, was employed to detect the presence of monoclonal antibodies, goat antirabbit IgG (whole molecule) HRP conjugate (Bio-Rad), diluted 1:1000 in blocking buffer (2 h at 37°C), was employed to detect rabbit antibodies.

The strips were then washed and subsequently stained with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (5-BICIP/NBT, Sigma) or 4-chloro-1-naftol (Sigma)-H₂O₂.

Patient sera used for *in vitro* characterization of the allergen content

Serum specimens were obtained from 15 atopic children (ages ranging from 3 months to 10 years, mean age: 4.5 years) diagnosed with IgE-mediated CMA. They showed a history of immediate reactions related to ingestion of CM (vomiting, diarrhea, dermatitis, bronchospasm, urticaria and rhinorrhea), specific IgE antibodies by immunoblotting and EAST, and positive skin prick test to CM proteins and common environmental allergens. Sera from 20 subjects (aged 2–6 years with no history of food allergy and no symptoms after ingestion of CM served as negative controls in these experiments.

All sera were obtained from the Hospital San Juan de Dios de La Plata (Argentina). This study was approved by the ethics committee of the Hospital San Juan de Dios de La Plata, and informed consent was obtained from the parents of all children participating in the study.

Sequential competitive ELISA

Polystyrene microtiter plates (NUNC, Maxisorp, Denmark) were coated with 1 µg/well of CMP extract, in 0.1 M carbonate/bicarbonate buffer, pH 8.5, for 12 h at 4°C. After washing with NaCl containing 0.05% (v/v) Tween 20 (NaCl-Tween 20), 200 µl/well of blocking buffer containing 5% horse serum in PBS, pH 7.4, was added and incubated for 2 h at 37°C. The wells were then washed with NaCl-Tween 20.

With regard to inhibition, preincubation of equal volumes of antigen (protein extracts from the different substitutes) and anti-CMP polyclonal antiserum – both dissolved in blocking buffer – was performed for 1 h at 37°C. Antiserum dilution was previously determined (1:140 000) from the titration curve.

A standard curve was obtained by preincubating CM samples of known protein content (0.5 ng/ml – 10 mg/ml) – as determined by the Lowry and Microkjeldahl methods –, with the antiserum.

A volume of 100 µl of each preincubated sample was transferred to the coated wells for competition with antigen in solid phase, by incubating for 1 h at 37°C. After washing with NaCl-Tween 20, 100 µl/well of goat antirabbit IgG horseradish peroxidase conjugate (Bio-Rad), diluted 1:3000 in blocking buffer, was incubated for 1 h at 37°C. Color was developed by adding a solution containing OPD (σ -phenylenediamine, ICN Biomedicals, Inc.) and 30% H₂O₂ in 0.1 M citrate-phosphate buffer pH 5.0 (2 µl/ml). The reaction was stopped by the addition of 50 µl/well of 4 N sulfuric acid, and the reaction product was quantified by reading the optical density (OD) at 490 nm.

Logit function (21) was applied to absorbance values to linearize it. Protein concentrations of samples were obtained by interpolating the lineal section of the curve.

To obtain the highest detectability of the assay, we tested different conditions: antiserum dilution, time and temperature of preincubation, coating-antigen concentration, time of the competition between immunocomplexes and coated antigen, and time of the antirabbit conjugate incubation.

Detection of specific IgE

Specific IgE in human sera was determined as previously described (7). Disks coated with proteins from the different extracts were incubated with 50 µl of serum for 6 h at room temperature. After thorough washing, monoclonal antihuman IgE alkaline phosphatase conjugate 1/6000 (Sigma) was added for 6 h at room temperature, and p-nitrophenyl phosphate (p-NPP, Sigma) was used as substrate. The reaction was stopped with 20 mM NaOH, and OD was measured at 405 nm.

To optimize the coating of paper disks, different protein concentrations of the CMP extract were tested. The concentration leading to no further increase of OD when analyzed with a pool of positive sera (1 mg/ml) was chosen as working condition. To determine whether this amount of coupled antigen was sufficient to bind all the specific IgE antibodies present in the serum, and to verify that no interference with specific IgG antibodies was occurring, a pool of positive sera was adsorbed with an antihuman IgG (γ -chain specific) monoclonal antibody, and then specific anti-CM IgE was measured as described above.

Test specificity was controlled by using serum samples with specific IgE against other allergens and total IgE level higher than 200 IU/ml. A serum with more than 20 000 IU/ml of total IgE was also tested. Background values were determined by analyzing sera from individuals of the control healthy group.

To measure specific IgE in classes, we obtained a calibration curve with a pool of anti-*Dermatophagoides* sp. sera (22).

Results

Protein composition of milk substitutes

Table 1 shows the protein content – as obtained with the chemical and immunochemical methods – of the different extracts analyzed. Values of protein concentration as determined by the Lowry method were higher than those obtained by the competitive ELISA method. In comparing the protein concentration between CM and CM substitutes, larger divergences were observed when the immunochemical method was used. As expected, lower values were observed with protein extracts obtained from the HF samples. When the pHF were analyzed by the Lowry method, a higher protein content was obtained than with eHF. However, when the ELISA inhibition test was employed, the BEBA HA2 sample rendered a lower specific content of CMP than that obtained with Pregestimil.

In Fig. 1, the protein staining of the tricine SDS-PAGE gels shows the main protein components of the different mammalian milks employed as antigens (lanes 1–5). Different CM components were tentatively identified by comparison with the relative MW determined by SDS-PAGE of different purified fractions of known MW. As can be seen, in the cow, sheep, goat, mare, and human milk profiles, the main bands corresponded to α -casein, β -casein, κ -casein, bovine seroalbumin (BSA), β -Lg, α -La, and high-molecular-weight (MW) casein aggregates. Sheep and mare milks contained lower quantities of α -casein. In human milk, weak bands were seen in the molecular-weight-zones corresponding to α - and β -caseins (lane 5). Maternal and mare milks showed a high number of components in a diffuse area, ranging from 5 to 13 kDa (lanes 4 and 5). Comparison of bands corresponding to whey components among the different mammalian milks showed some relative differences: higher amounts of α -La could be detected in sheep, mare, and human milks, while a strong band corresponding to β -Lg was observed in the sheep and mare milk protein profiles. Regarding the HF (lanes 6–12), some pHF presented a larger amount of polypeptides of

Table 1. Protein contents of milk substitutes

Dairy product	Lowry method		Competitive ELISA μg/ml*	ELISA/Lowry %
	mg/ml	g/100 g product		
CM	5.1	35	4730	92.7
Sheep milk	20.3		601	2.96
Goat milk	18.4		590	7.02
Mare milk	15.4		3.49	0.022
Human milk	9.26		1.93	0.028
NAN HA	1.5	45	801	53.4
Kas 1000	3.2	32	180	5.6
Pregestimil	1.35	5.32	0.34	0.025
BEBA HA 1	1.32	8.80	3.18	0.24
BEBA HA 2	1.28	8.53	0.013	0.00101
HUMANA HA	1.22	8.13	2.26	0.18
APTAMIL HA	0.74	4.93	9.31	1.26
Neocate	5.25		0.011	0.002

* This immunoenzymatic method detects presence of CMP. CMP were used as coated antigens, and each dairy product was employed as inhibitor.

low MW, especially Kas 1000 (lane 7) and HUMANA HA (lane 10). Sample extracts from BEBA HA 1 (lane 8) and HUMANA HA (lane 10) showed a band of 67 kDa that corresponded to BSA or casein aggregates. Low MW-components in the zone of 10 kDa could be detected in samples from BEBA HA 2, HUMANA HA, and APTAMIL HA (lanes 9–11). These peptides are often eluted when conventional SDS–PAGE is used to separate protein fractions. It may be noted that HUMANA HA and APTAMIL HA (lanes 10 and 11) present a weak band with MW similar to β -casein. The pHF Kas 1000 (lane 7) contained α - and β -casein, as well as peptides derived from casein hydrolysis. When the NAN HA sample was analyzed, bands corresponding to intact whey (α -La and β -Lg) and casein components (α -

Table 2. Serum specific IgE detected by EAST

Dairy product	Specific IgE (class)						
	"Pool" of positive sera	"Pool" of negative sera*	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5
CM	3	0	2	2	1	4	4
Sheep milk	2	0	2	0	2	3	2
Goat milk	2	0	0	0	0	3	2
Mare milk	2	0	2	3	2	2	3
Human milk	3	0	1	2	2	2	2
NAN HA	2	0	2	1	0	2	2
Kas 1000	3	0	1	1	1	3	1
Pregestimil	3	0	0	0	0	1	0
BEBA HA 1	3	0	2	1	0	0	0

* OD of class 0 serum was around 0.3.

and β -casein) were revealed (lane 6). However, no visible bands could be detected either in the amino-acid formula Neocate (lane 13), or in the casein hydrolysate Pregestimil (lane 12), suggesting a high degree of protein hydrolysis.

Antigenicity and allergenicity of milk substitutes

Table 2 shows the results of the EAST obtained from five representative sera out of the 15 sera analyzed. As demonstrated by these typical examples, all substitutes except Neocate contain components that could be bound by serum IgE. These results suggest that even small peptides, not detected by the tricine SDS–PAGE technique, contain epitopes that could be recognized by IgE antibodies. Since these patients have been exposed only to CM, the results obtained when other mammalian milk proteins were employed as antigen suggest that these mammalian systems contain components with

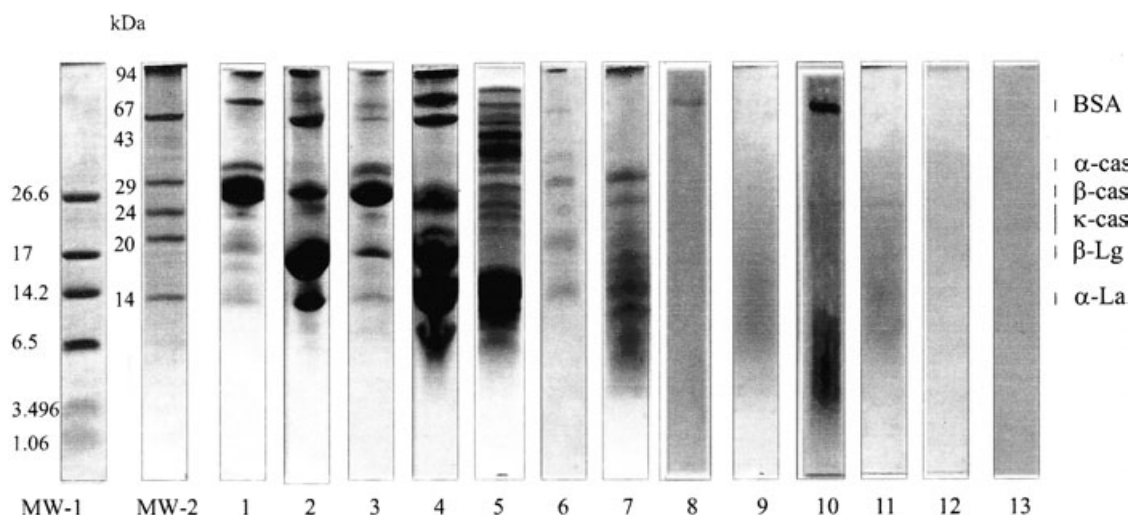


Figure 1. Tricine SDS–PAGE analysis of protein extracts from mammalian and formula milks under reducing condition. Amount of protein loaded is indicated in parantheses. MW-1 and -2) molecular weight markers; lane 1) CM (13 μg); 2) sheep milk (8.6 μg); 3) goat milk (7.9 μg); 4) mare milk (8.4 μg); 5) human milk (4.2 μg); 6) NAN HA (3.4 μg); 7) Kas 1000 (7.3 μg); 8) BEBA HA 1 (10 μg); 9) BEBA HA 2 (9.8 μg); 10) HUMANA HA 1 (9.6 μg); 11) APTAMIL HA 1 (5.6 μg); 12) Pregestimil (6.4 μg); 13) Neocate (5.2 μg). Lanes 1–5 were stained with Coomassie brilliant blue; lanes 6–13 were stained with silver. Various individual components were identified by comparison with purified fractions.cas: casein.

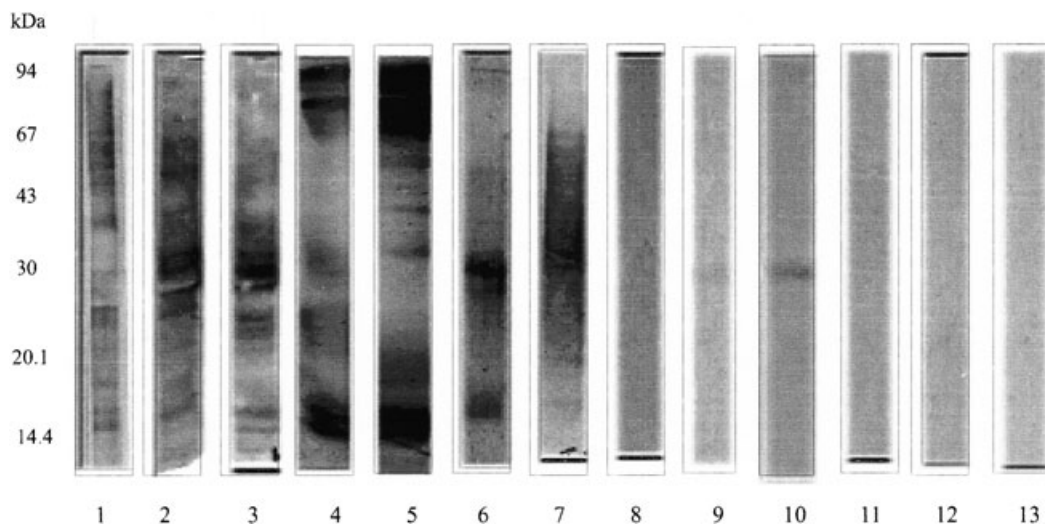


Figure 2. Antibody binding to milk-substitute fractions isolated on SDS-PAGE immunoblots for CMP-specific polyclonal antiserum. 1) CM; 2) sheep milk; 3) goat milk; 4) mare milk; 5) human milk; 6) NAN HA; 7) Kas 1000; 8) Pregestimil; 9) BEBA HA 1; 10) HUMANA HA 1; 11) BEBA HA 2; 12) APTAMIL HA 1; 13) Neocate.

cross-reactive epitopes. Differences in individual recognition of the extracts may be explained by the great variability in specificity and affinity of serum antibodies from different individuals. When sera from the non-allergic subject group were analyzed, background values were obtained (OD around 0.3), corresponding to class 0 results.

The specificity of antibodies, as well as the detection of antigenic and allergenic protein determinants, was analyzed by immunoblotting with a polyclonal antiserum, monoclonal antibodies specific to CM caseins, and sera from CMA patients. Each set of immunoblots was controlled for nonspecific binding by a blot without serum or with sera from subjects of the control group.

When protein extracts from cow, sheep, and goat milk were used as antigen, and anti-CM polyclonal antiserum was employed as the first antibody, similar reactivities were obtained, (Fig. 2, lanes 1–3). The most intense bands corresponded to α - and β -casein, while β -Lg, α -La, and the glycosylated κ -caseins could also be detected in several bands. Bands in the upper zone of the gel, corresponding to casein aggregates, could also be observed. Protein extract from mare milk (lane 4) revealed strong bands in the high-MW zone and, in the region corresponding to α -La. Only weak reactivities to casein components (α - and β -casein) were seen. In the human milk sample (lane 5), bands corresponding to α -La and high-MW aggregates were observed with higher intensity. Faint bands in the region of caseins were seen. Reactivities to CM caseins could also be detected in the whey- and casein-based pHF (lanes 6, 7, and 10). In the NAN HA hydrolysate (lane 6), a 15-kDa component, not revealed by the tricine SDS-PAGE method, showed an intense reactivity, while with Kas 1000 sample (lane 7), a wide zone of diffuse staining was observed. Conversely, no reactivities were revealed when either the amino-

acid-based formula (lane 13), the casein-based eHF (lane 8), BEBA HA 1 (lane 9), BEBA HA 2 (lane 11), or APTAMIL HA (lane 12) were used as antigen.

A pooled serum prepared with sera class 4 or higher to CMP, as determined by EAST, was employed in immunoblotting to reveal the presence of allergenic components. The IgE immunoblotting reactivities shown in Fig. 3 revealed that almost all the main whey and casein components of cow, sheep, and goat milk contained allergenic determinants (lanes 1–3). The highest reactivities were observed in the α -casein, β -casein, and casein aggregates regions. Less intense bands were revealed when proteins from mare milk were coupled to the solid phase (lane 4), although high-MW and whey components would contain epitopes that can be bound by IgE. In the human milk sample, components of around 70 kDa of relative MW could be bound by serum IgE, and casein aggregates would be implicated in this reactivity (lane 5). The pHF NAN HA and Kas 1000 contained IgE reactive components with relative MW corresponding to the caseins (lanes 6 and 7). The 15-kDa component in the NAN HA sample recognized by the polyclonal antiserum (Fig. 2, lane 6) was not bound by serum IgE. In the immunoblotting corresponding to BEBA HA 1 and HUMANA HA (lanes 9 and 10), a region of diffuse reactivity between 20 and 67 kDa was observed. Bands corresponding to β -casein could also be observed in these samples. In contrast, no reactivities could be detected with either the pHF BEBA HA 2 and APTAMIL HA (lanes 11 and 12), the eHF Pregestimil (lane 8) or the amino-acid-based formula Neocate (lane 13) employed as antigens. Immunoblots developed with sera from nonallergic subjects showed no reactivity to any of the antigens employed (data not shown).

On immunoblots to the different protein extracts, monoclonal antibodies were useful to identify compo-

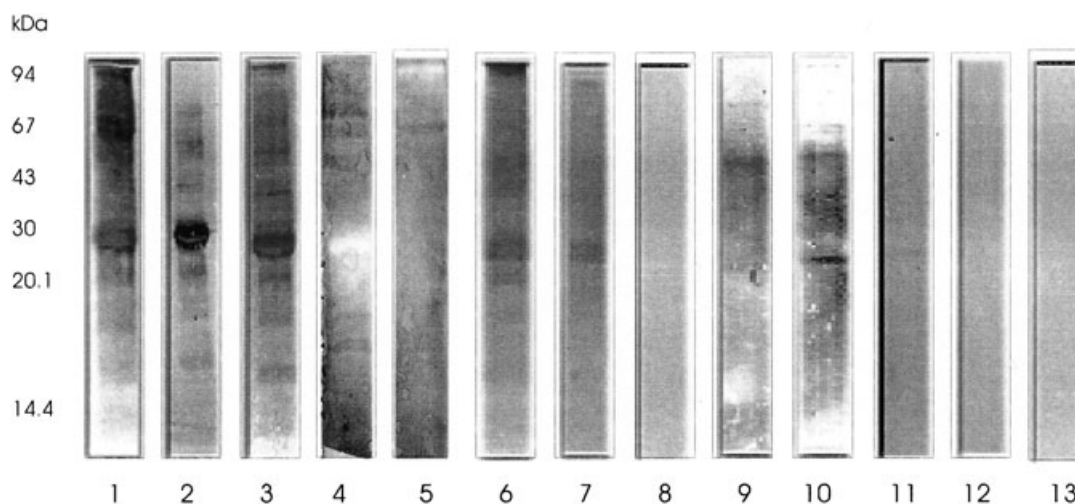


Figure 3. Antibody binding to milk-substitute fractions isolated on SDS-PAGE immunoblots for sera from CM-allergic patients (serum pool). 1) CM; 2) sheep milk; 3) goat milk; 4) mare milk; 5) human milk; 6) NAN HA; 7) Kas 1000; 8) Pregestimil; 9) BEBA HA 1; 10) HUMANA HA 1; 11) BEBA HA 2; 12) APTAMIL HA 1; 13) Neocate.

nents with different relative MW (Fig. 4). These antibodies could recognize cross-reactive epitopes present in α -, β -, and κ -casein from different mammalian milks (lanes 1–4). Reactivities in the regions where casein monomers and high-MW aggregates migrate could be detected with the cow, sheep, goat, and mare samples (lanes 1–4). Immunoblots corresponding to human milk sample (lanes 5) revealed different bands in the regions of high MW with these monoclonal antibodies. Even the 70-kDa band observed in the IgE immunoblotting was also detected by the three monoclonal antibodies, confirming that in this region casein aggregates are present. Casein monomers were also detected in hydrolyzed formulas, even in the whey-based hydrolyzed formula NAN HA, where casein components were not supposed to be present (lane 6A and C). The 15-kDa peptide detected by the polyclonal antiserum in the NAN HA extract (Fig. 2, lane 6), but not by the human serum IgE, was derived by hydrolysis from β -casein (Fig. 4, lane 6B). Furthermore, this whey-based formula contained high-MW casein aggregates. Peptides derived from caseins were revealed in the pHF (lanes 6 and 7A–C). On immunoblots of the moderately hydrolyzed casein substitute Kas 1000 (lanes 7), the monoclonal antibodies detected α -, β -, and κ -caseins not only as monomers, but also as high-MW aggregates, and as peptides that migrate in a diffuse area of different MW. Neither the casein-based eHF Pregestimil; the pHF BEBA HA 1 and BEBA HA 2, HUMANA HA, and APTAMIL HA samples; nor the amino-acid-based formula Neocate contained components with epitopes that could be bound by the three mentioned antibodies, as shown in lane 8.

Discussion

In this study, we have investigated the presence of

residual antigenic and allergenic components in different milk substitutes employed in the prevention and treatment of CMA.

During the CM elimination period, substitution is necessary to meet the patient's nutritional requirements and to maintain normal growth. Alternative foods include milks from different species, soy-based formulas, CMP-hydrolyzed formulas, and amino-acid-based formulas. Extensively hydrolyzed formulas differ from regular formulas in smell, taste, osmolarity, and cost. To overcome these problems, less hydrolyzed formulas have been introduced, although peptidic components may retain allergenic epitopes, and hence, residual allergenicity.

By using a CM-specific antiserum and casein-specific monoclonal antibodies in immunoblotting and ELISA, we could detect and identify residual components in CM hydrolysates and cross-reactive proteins in mammalian milks. Moreover, the ability of these components to be bound by human IgE was evaluated by immunoblotting and EAST (Table 2 and Fig. 3). These results may explain the occasional occurrence of reactions to casein- or whey-based hydrolyzed formulas associated with their intake (23, 24).

Plebani et al. (25) employed an ELISA inhibition test to demonstrate the presence of residual casein components in whey-based HF. However, these authors could not detect residual antigenic components in casein eHF or amino-acid-based formulas, although anaphylactic reactions in very sensitive allergic infants were reported (24, 26). Our results show the presence of casein-processed or unprocessed components in casein-based hydrolysates, and of casein fractions in a whey-based hydrolyzed formula, as contaminants. These results indicate the unsatisfactory technological separation of caseins from whey proteins, as has also been reported by other authors (27, 28), and the difficulty in the elimin-

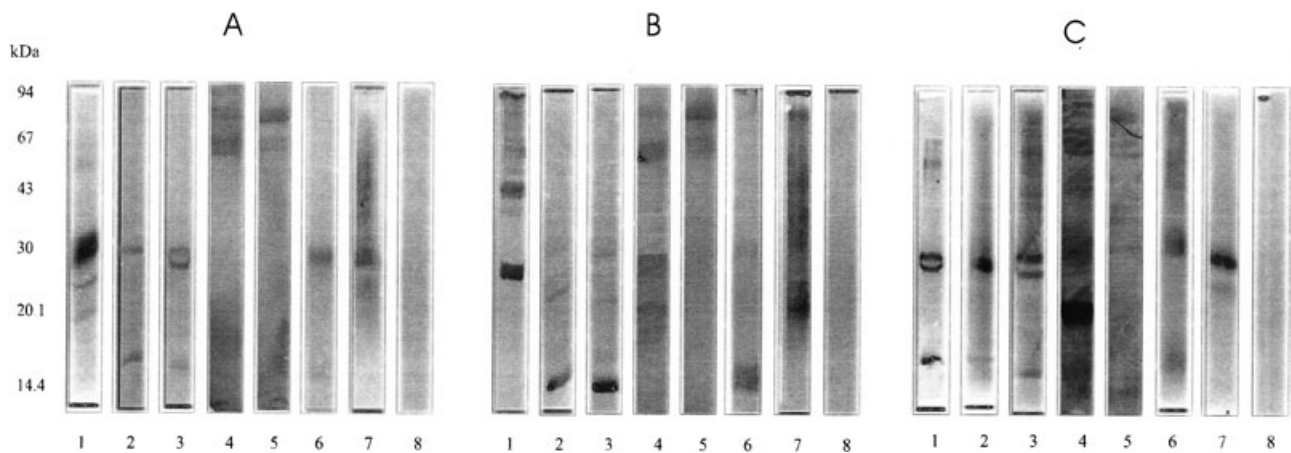


Figure 4. Antibody binding to milk-substitute fractions isolated on SDS-PAGE immunoblots for monoclonal antibodies specific to CM caseins. A) Immunoblots performed with mAb 1D5-I (specific for α -casein); B) Immunoblots performed with mAb 2A1-I (specific for β -casein); C) Immunoblots performed with 3B5-I (specific for κ -casein). 1) CM; 2) sheep milk; 3) goat milk; 4) mare milk; 5) human milk; 6) NAN HA; 7) Kas 1000; 8) Pregestimil, BEBA HA 1, BEBA HA 2, HUMANA HA 1, APTAMIL HA 1, Neocate.

ation of immunoreactive epitopes present in casein aggregates after enzymatic treatment. For example (Fig. 4), it was possible to detect reactivities of casein-specific monoclonal antibodies to intact α -casein, and processed β - and κ -casein in NAN HA. However, 2A1-I did not react with β -casein from HUMANA HA and BEBA HA1, while the polyclonal antiserum and serum IgE antibodies could detect a component with the MW of this component.

By ELISA (Table 1) and EAST (Table 2), residual antigenic and allergenic peptide-containing immunoreactive epitopes could be detected in the eHF Pregestimil. Restani et al. (27) enriched protein extracts by precipitation to detect the presence of peptides or intact proteins in eHF. In our conditions, they could be detected by fixing to a solid phase, such as special plastic or CNBr-activated disks. As in other investigations (29), ELISA was useful to detect the presence of contaminants in HF.

The pHF BEBA HA 2 analyzed by the ELISA inhibition test yielded lower quantities of proteins than those obtained with the eHF Pregestimil, although the total protein content was higher in the pHF. This result could be explained by the different specificities of the antibodies in the polyclonal specific antiserum. Depending on the immunized animal, different specific recognition patterns may be obtained. Therefore, to develop a quantitative inhibition ELISA, several specific antisera should be pooled. For this reason, the inhibition ELISA was employed just for detection of specific CMP. The same results were observed (Table 2) when different sera from CMA patients were employed as the source of IgE. The results shown here suggest differences in the individual patterns of sensitization to the various CMP.

In the region of 66 kDa, it was possible to detect a band by SDS-PAGE in BEBA HA 1 and HUMANA HA samples. This component reacted only with IgE

antibodies. As judged by its relative MW, it corresponded to either BSA or casein aggregates. Since the former would be hydrolyzed during the technological treatment, we conclude that casein aggregates were responsible for binding serum IgE antibodies.

In accordance with the study of Niggemann et al. (30), the partially hydrolyzed whey and casein formulas contained components able to be bound by IgE. They also found that the whey-based pHF showed higher protein content and residual allergenicity than any other hydrolyzed formulas. The dissimilar protein and reactivity profiles of the different whey-based pHF analyzed (NAN HA, BEBA HA 1, and BEBA HA 2) suggest different technical processing of CMP.

Milks from species other than cow contain components that share identity with the main allergenic proteins of CM, resulting in cross-reactivity and allergenicity (8, 9). We could demonstrate this by using sera from patients that had been only exposed to CM (Table 2 and Fig. 3). IgE reactivities to casein and whey components from different mammalian milks suggest the presence of cross-reactive epitopes in these fractions. Besides, the results obtained employing a rabbit antiserum-specific to CMP and monoclonal antibodies against CM caseins, as shown in Table 2 and Figs. 2–4, can be justified by the sequence homology and by the presence of similar or identical epitopes in the main antigenic proteins of these milks. Reactivities observed at high-MW zones in mare and human milk with monoclonal antibodies suggest that conformational epitopes are created when casein fractions are aggregated spontaneously. Among the mammalian milks analyzed, mare milk was found to be the least reactive substitute, with these *in vitro* tests. By ELISA inhibition, the polyclonal antibody binding to the CM-coated microtiter plates was weakly inhibited with extracts from mare and human milks (percentage not shown). This result could be explained by the

differences from CM in the amino-acid sequences of some mare milk proteins. The protein composition of mare milk is much more similar to human milk than to CM; mare milk is particularly rich in whey proteins, and casein components occur in lower quantities than in CM (Fig. 1). Other authors found similar results by *in vitro* and *in vivo* tests, and they suggest that mare milk would be a good substitute for CM in CMA patients (30).

Analyzing sera from patients not previously fed with any goat milk-containing food, Businco et al. (31) have also reported the highly allergenic behavior of caseins from goat milk. For this reason, they recommend that goat milk not be given to CM-allergic patients. In contrast, Restani et al. (9) suggest that goat milk would be well tolerated by some CMA patients.

As for the sheep milk sample analyzed, the protein profile obtained by tricine SDS-PAGE under reduced conditions showed higher amounts of whey components, basically β -LG, than that observed by other authors (9).

The indiscriminate labeling of infant formulas as hypoallergenic, merely on the basis of the degree of hydrolysis of CMP, poses the risk of adverse reactions in CM-allergic individuals. Through the detection of residual native or processed components by these methods, we could predict the potential allergenicity of the substitute. Moreover, residual proteins or peptides could be identified by using monoclonal antibodies. We have employed casein-specific monoclonal antibodies, since caseins have been characterized as the main allergenic fraction of CM (11).

Human milk constitutes the optimal source of nutrition for healthy and allergic infants. Since dietary native proteins have been detected in breast milk and serum (32), it is important to avoid the intake of CMP by the mother during the lactation period. Caseins from CM or synthesized by the human breast gland (β - and κ -caseins) tend to form high-MW aggregates that could be detected by the different antibodies used (Figs. 2–4). These aggregates retain the capacity to bind serum IgE

(Fig. 3). When healthy infants are weaned from breast milk, the current formulas ensure normal growth. There are minor differences in growth between breast-fed and formula-fed healthy infants. However, there are many differences between the composition of formulas and human milk with respect to several substances that may affect growth and host defense, and even differences in protein and allergen composition. Therefore, efforts should be directed to improve the quality of formulas for infants who are premature, allergic, or exhibiting any other pathophysiologic condition that may lead to a chronic disease or allergic reaction.

In order to obtain complete information about the antigenic and allergenic composition of CM substitutes, different immunochemical methods and several antibodies (polyclonal antisera, monoclonal antibodies, and human sera) should be employed.

In conclusion, our results suggest that the least antigenic and allergenic substitutes seems to be the amino-acid-based formula Neocate. Mare milk (among mammalian milks) and the casein-based eHF Pregestimil proved to be the least antigenic and allergenic substitutes with the *in vitro* tests here employed.

CMA is not an isolated phenomenon, and it may constitute the starting point of an atopic condition. Thus, prevention may not only be useful to avoid a transient adverse reaction, but may also be a positive influence on the allergic condition. Hence, an early diagnosis of CMA and an effective treatment may ensure the normal growth of infants. To achieve this goal, immunochemical methods constitute a useful tool to determine the residual allergenicity of CM substitutes.

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