

Use of immunoblotting and monoclonal antibodies to evaluate the residual antigenic activity of milk protein hydrolysed formulae

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Summary

Background Partial and extensive hydrolysed protein formulae have been developed to lower or eliminate the antigenicity of milk proteins. Although normally well tolerated, extensive hydrolysates have been reported to induce serious allergic reactions in very sensitive children. Moreover, clinical practice has often raised concern about the role of partial hydrolysates in cow's milk allergy prevention.

Objective Starting from these considerations, we used anti-casein monoclonal antibodies to evaluate the presence of residual antigenic activity in both partially and extensively protein hydrolysates.

Methods Electrophoretic analyses associated with immunoblotting technique were performed using nine protein-enriched commercial formulae.

Results The presence of different amounts of residual intact cow's milk proteins and/or polypeptidic material with conserved antigenic activity (according to the extensive or partial hydrolysis) was verified in most milk-based samples considered.

Conclusion The use of monoclonal antibodies and immunoblotting could be useful for the quality control of commercial 'hypoallergenic' formulae.

Keywords: cow's milk allergy, hydrolysed protein formulae, immunoblotting, monoclonal antibodies

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Introduction

Cow's milk allergy (CMA) affects about 3% of the infant population. Cow's milk contains more than 30 proteins and many of them can produce IgE-mediated responses in genetically predisposed children. In the absence of maternal milk, soy and protein hydrolysed formulae are the most widely used alternatives for allergic subjects.

The hydrolysed formulae have been developed to lower or eliminate the antigenicity of milk proteins (extensive

hydrolysates) or to reduce the risk of sensitization (partial hydrolysates).

Indications for the use of infant formulae containing partially hydrolysed milk proteins have not yet been defined. These formulae have been noted in some studies [1,2] as useful in the prevention of allergy in infants with a high risk of atopy; however, other authors have reported no significant difference in the clinical prevalence of symptoms in groups fed an adapted or partially hydrolysed formula [3,4].

Different studies have reported a residual antigenic activity in both partially and extensively hydrolysed formulae [5–8]; moreover, several clinical studies have shown that anaphylactic reactions occur in children fed extensive

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hydrolysates [9,10]. To demonstrate the possible antigenicity of these products, we have shown the presence of residual intact proteins, which were able to bind specific circulating IgE as assessed by the immunoblotting technique [11].

In this paper, thanks to the use of monoclonal antibodies directed against α , β and κ caseins, we confirm previous results and show that these reagents can be useful to evaluate the residual antigenic activity of hydrolysed formulae.

Materials and methods

Purified milk proteins

Purified cow's milk proteins were purchased from Sigma Chemical Co. (St Louis, MO, USA) at the highest available purification level and with the following chemical characteristics: α -casein (lyophilized powder, chromatographically purified; approximately 85% by electrophoresis); β -casein (lyophilized powder; minimum 90% by electrophoresis); κ -casein (lyophilized powder; minimum 80% by electrophoresis); α -lactalbumin (lyophilized powder; approximately 85% by electrophoresis); β -lactoglobulin (3 $\times$ crystallized and lyophilized; approximately 95% by Lowry and biuret methods); and bovine serum albumin (BSA; essentially globulin free; lyophilized powder; approximately 99% by agarose electrophoresis).

We prepared chemically enriched $\beta + \gamma$ -caseins ourselves from a total casein extract [12], following the fractionated precipitation method of Tripathi & Gehrke [13]. Purified proteins were suspended in sample buffer (see below) at a final concentration of 0.5–1 mg/mL.

Milk formulae

Samples used in this study included one formula for newborns, containing cow's milk proteins, one formula containing isolated soy proteins; and seven (three partially and four extensively) hydrolysed formulae, with the following characteristics: Humana1 (HM1; Humana, Westfalen Eg Herford, Germany; a conventional cow's milk-based formula, containing 60% and 40% caseins and whey proteins, respectively); Isomil (IS; Abbott, North Chicago, IL, USA; a soy formula); Alfaré (AL; Nestlé, Nuhspeet, The Netherlands; an extensive whey hydrolysate); Aptamil HA (AHA; Milupa, Friedrichsdorf, Denmark; a partial casein and whey hydrolysate); Nidina HA (NHA; Nestlé; a partial whey hydrolysate); Nutrilon Pepti (NTL; Nutricia, Cuyk, The Netherlands; an extensive whey hydrolysate); Pepti Junior (PJ; Nutricia; an extensive whey hydrolysate with short-chain peptides); Pregestimil (PRS; Mead Johnson; an extensive casein hydrolysate); and Vivena HA (VHA; Diet-erba, Plada, Latine, Italy; a partial whey hydrolysate).

Protein precipitation with trichloroacetic acid (TCA)

Both hydrolysed and non-hydrolysed formulae were weighed in 50-mL centrifuge tubes and suspended in TCA (20% final) at the following concentrations: 40 mg/mL for hydrolysed and 6 mg/mL for non-hydrolysed samples. After 30 min at 4°C, samples were centrifuged (J2-21 Centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA) for 30 min at 4°C and 10 000 r.p.m. 12 100 g. Pellets were washed three times with acetone, and finally freeze-dried (Modulyo, Edwards Alto Vuoto, Trezzano sul Naviglio, Italy). The lyophilized products were suspended in sample buffer (3 mg/mL) (see below) and loaded onto the electrophoretic gel.

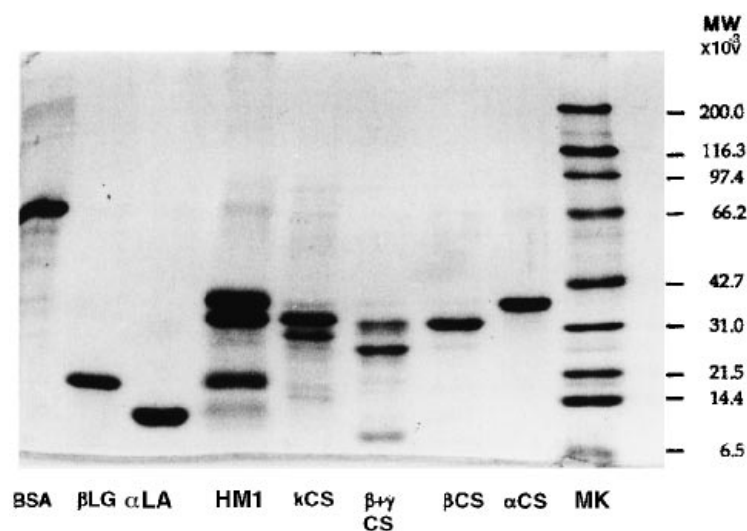


Fig. 1. SDS-PAGE of milk proteins. CS, casein; α LA, α -lactalbumin; β LG, β -lactoglobulin; BSA, bovine serum albumin; HM1, Humana 1; MK, MW standard solution.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

All chemicals and instruments used in the electrophoretic analyses were purchased from Bio-Rad (Richmond, CA, USA).

The composition of electrophoretic gels were: *gradient running gel*: 9–10% acrylamide; 0.08–0.17% bis-acrylamide; 0.36 M Tris (hydroxymethyl) aminomethane (TRIS)-HCl buffer, pH = 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N',N'-tetramethylethylenediamine (TEMED). Bromophenol blue was added to the gel as a run marker. *Stacking gel*: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M Tris-HCl buffer, pH = 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% TEMED. *Running buffer*: had the following composition: 25 mM TRIS, 0.19 M glycine and 0.1% SDS (w:v), pH = 8.8.

Before performing the electrophoretic run, samples were suspended in sample buffer (0.25 M Tris-HCl buffer, pH 6.8, containing 7.5% glycerol, 2% SDS and 5% β -mercaptoethanol) and heated for 10 min at 100°C. After the electrophoretic run (90 V at room temperature, for approximately 6 h), gels were dyed with Coomassie Brilliant Blue G-250 according to the method of Neuheff *et al.* [14]. Molecular weight (MW) standard solution (Broad range) was purchased from Bio-Rad and contained: rabbit skeletal muscle myosin (200 000 MW); *Escherichia coli* β -galactosidase (116 250 MW); rabbit muscle phosphorylase b (97 400 MW); BSA (66 200 MW); hen egg white ovalbumin (42 700 MW); bovine carbonic anhydrase (31 000 MW); soybean trypsin inhibitor (21 500 MW); white hen egg lysozyme (14 400 MW); bovine pancreas aprotin (6500 MW).

Immunoblotting

After SDS–PAGE, transfer of proteins from the gel onto a polyvinylidene difluoride (PVDF; Immobilon P; Millipore, Bedford, MA, USA) membrane was achieved by electrophoretic elution (wet transfer). The transfer buffer was Tris 25 mM, glycine 193 mM and methanol 20%. To verify the protein transfer and the precise protein position, a solution of 0.2% Ponceau S (in 3% TCA and 3% sulphosalicylic acid) was used. After the destaining, PVDF membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin (Bio-Rad) solution (in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton-X) to prevent non-specific adsorption of the immunological reagents. The membrane was then immersed in 10 ml of 0.25% gelatin solution, containing 30 μ l of monoclonal antibodies. Antigen–IgG complex was detected using goat anti-mouse IgG antibodies (Sigma Chemical Co.) 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).

Preparation of anti-casein monoclonal antibodies

Anti-casein monoclonal antibodies were prepared according to the method of Galfré *et al.* [15], with some modifications.

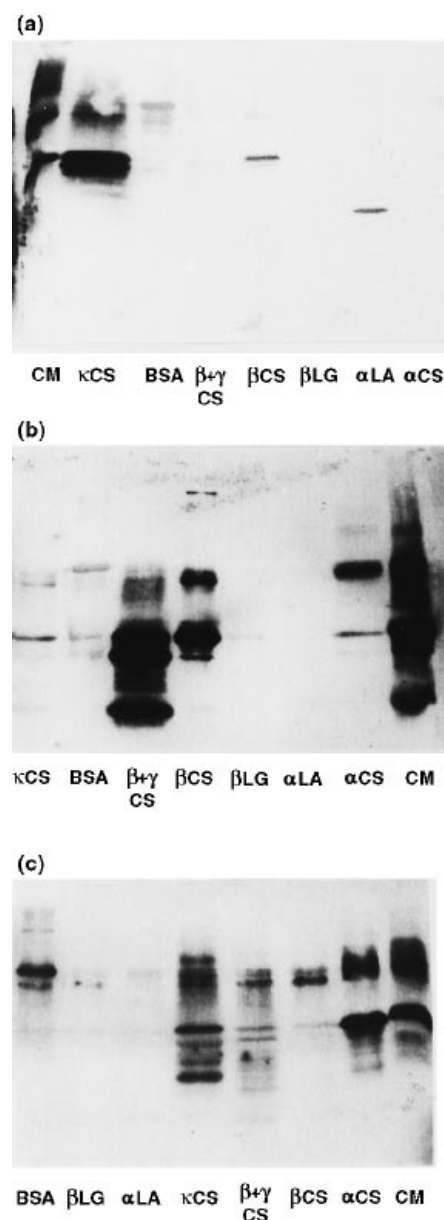


Fig. 2. Specificity of anti-casein monoclonal antibodies used in this study: Mo50 (a), Mo71 (b) and Mo157 (c) (CM = cow's milk; other abbreviations as in Fig. 1).

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.), 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, 1000 MW. 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 anti-casein 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 casein-specificity of the monoclonal antibodies harvested from the peritoneal fluid was then assayed and three monoclonal antibodies (Mo50, Mo71 and Mo157) were selected for the present study.

Results

Specificity of monoclonal antibodies Mo50, Mo71 and Mo157 assayed by immunoblotting

The specificity of the monoclonal antibodies used in this study was evaluated by immunoblotting. In Fig. 1, the electrophoretic profile of purified cow's milk proteins is shown. As described in our previous paper [11], the κ -casein shows some heterogenic fraction (due to the different quantity of bound carbohydrates); in the $\beta + \gamma$ -casein sample, three major bands were visible, from the gel start they correspond to β , γ_1 and γ_{2-3} caseins.

The specificity of the monoclonal antibodies is shown in Fig. 2a (Mo50), b (Mo 71) and c (Mo157). Mo50 (Fig. 2a) was highly specific for κ -casein; weak responses were found

versus BSA and α -lactalbumin. A residual quantity of κ -casein was visible in purified β -casein.

Specificity of Mo71 is represented in Fig. 2b: this antibody was highly specific for β - and γ -caseins; traces of these proteins were also visible in some other purified proteins (α -casein, κ -casein and BSA).

Mo157 reacted specifically with α - and κ -caseins; in purified β - and γ -caseins it was possible to note the presence of residual amounts of α - and κ -caseins. When compared to Mo50, Mo157 recognized different epitopes of κ -casein, as shown by the different specificity in Fig. 2a, c.

Immunoblotting of TCA-precipitated hydrolysed formulae

After enrichment of the protein fraction by TCA precipitation, each milk formula considered here showed protein bands in SDS-PAGE (Fig. 3). As expected, partially hydrolysed formulae presented a higher quantity of protein material, distributed in a wide range of molecular weights. On the other hand, extensively hydrolysed products showed a lower residue, often concentrated in only a few bands.

When immunoblotting was performed, well-defined antibody-antigen complexes were often observed for both partial and extensive hydrolysates.

The reactivities obtained in immunoblotting are shown in Fig. 4. Although all nine formulae were tested with the three monoclonal antibodies, only significant positive responses are illustrated in Fig. 4. Mo50 (Fig. 4a), which was highly specific for κ -casein, identified antigenic material in most hydrolysed formulae shown in this figure. No reactivity against the extensive hydrolysate NTL and an aspecific reactivity to NHA were observed.

Mo71 (Fig. 4b), highly specific for $\beta + \gamma$ -caseins, was found to react positively with different commercial products; in particular, residual caseins were visible in extensive whey hydrolysates (AL and NTL), while no reaction was detected with extensive casein hydrolysate (PRS). A

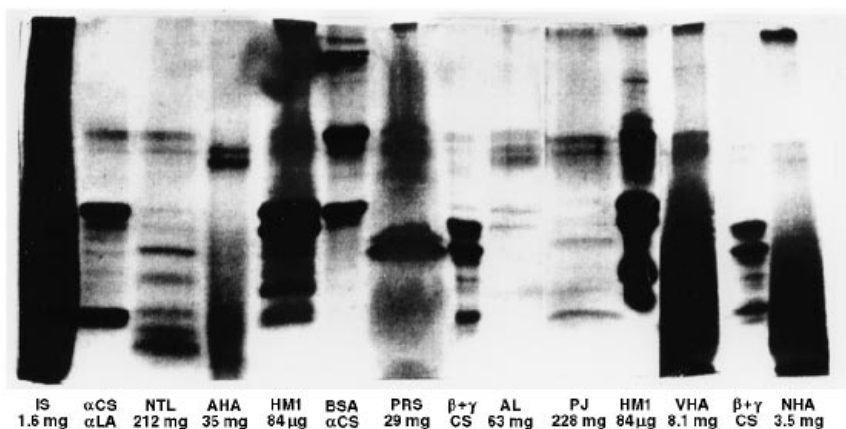


Fig. 3. SDS-PAGE of hydrolysed formulae after protein enrichment by TCA precipitation (abbreviations are specified in the Materials and Methods section). The quantities indicated below represent mg of formula as such, before TCA precipitation, loaded onto the gel.

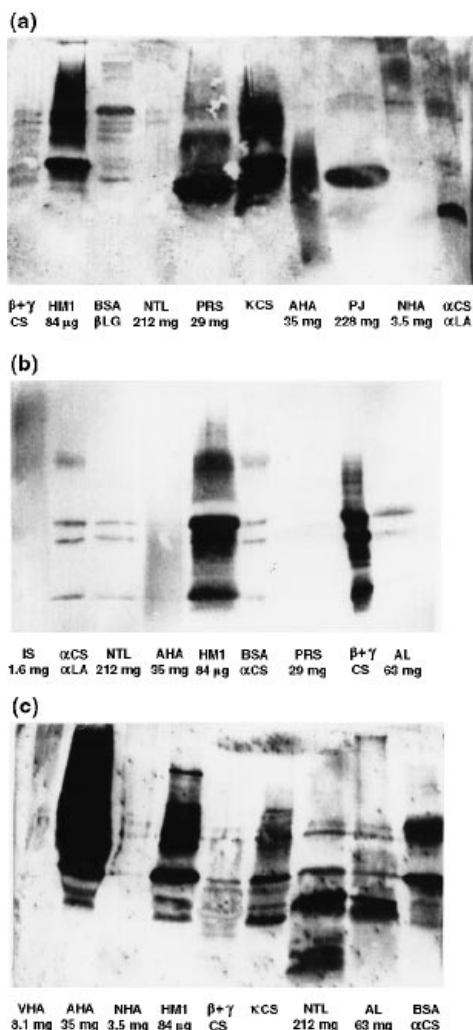


Fig. 4. Immunoblotting of protein-enriched hydrolysed formulae. Monoclonal antibodies used were Mo50 (a), Mo71 (b) and Mo157 (c). Only positive responses are shown. Abbreviations are specified in the Materials and Methods section. The quantities indicated below represent mg of formula as such, before TCA precipitation, loaded onto the gel.

diffused area was observed in the partial hydrolysed formula (AHA) where polypeptidic fragments, deriving from the $\beta + \gamma$ caseins, may be present. Only unspecific reactions were present in the soy formula (IS).

Mo157 (Fig. 4c), highly specific for α - and κ -caseins, recognized antigenic material in partial (AHA) and extensive (AL and NTL) hydrolysates. Very low reactions were visible in two other partial hydrolysates (NHA and VHA) but it is important to stress that the quantity of formula (as reported below each line) that could be loaded onto the gel varied according to the partial or extensive hydrolysis.

Discussion

Partial hydrolysates are sometimes suggested for the prevention of sensitization in at-risk subjects. On the basis of our results, this attitude is questionable because these preparations can contain a relatively high amount of residual antigenic activity. As an example, shown in Fig. 4a, c, it was possible to detect a strong reactivity of Mo50 and Mo157 to AHA; on the other hand, only weak reactivity was shown against NHA. These results could be due to: (a) the different quantity of formula that can be loaded onto the gel (35 versus 3.5 mg); (b) the different degree and/or quality of protein hydrolysis. The different degree of hydrolysis in different commercial partial hydrolysates could be responsible for the contrasting clinical data obtained with these products in milk allergy prevention.

Extensively hydrolysed protein formulae are widely used in the diet of children with cow's milk allergy. Normally well tolerated, these products have been shown to induce serious clinical reactions (reaching anaphylaxis) in those subjects who present a higher sensitivity.

There are two possible explanations for the clinical reaction observed: (a) hydrolysed formulae contain a low amount of residual intact proteins that are able to stimulate immunological reactions in very sensitive subjects; (b) peptidic material coming from the hydrolysis of cow's milk proteins could maintain the antigenic activity. In a previous paper [5], we showed that IgE-antigen complexes can be obtained in immunoblotting using the sera of children with RAST class ≥ 3 . Most positive reactions were associated with residual amounts of intact proteins; only in a few cases was polypeptidic material responsible for binding circulating IgE.

The presence of residual antigenic material in extensive hydrolysates (though very slight) raises concern about their suitability for the diet of children with severe cow's milk allergy. Since hydrolysed formulae are so far the only alternative protein sources for some polyallergic subjects (reactive to milk, soy, meat, etc.), an improved quality control is necessary to evaluate the residual antigenic activity in these preparations and thus reduce the risk of allergic reactions.

In this paper, we present data obtained using monoclonal antibodies specific for α -, β - and κ -caseins. Residual antigenicity was observed with most hydrolysed formulae tested and with all monoclonal antibodies. Residual intact proteins are often responsible for this activity but, in partial hydrolysates, polypeptidic material may also be involved.

Unexpectedly, positive responses to residual intact proteins were particularly high in extensive hydrolysates; this could be associated with: (a) the different amount of formula that can be loaded onto the gel, 3.5–35 mg for partial hydrolysates and 29–228 mg for extensive hydrolysates; (b)

the presence in partial hydrolysates of a high quantity of polypeptidic material that overlaps and does not always allow identification of residual intact proteins in an electrophoretic run.

It seems important to point out that monoclonal antibodies were able to identify caseins in whey protein-hydrolysed formulae (i.e. α - and β -casein in AL), showing that the technological separation of caseins from the whey proteins may not always be satisfactory. This suggests that whey protein hydrolysates cannot always be considered safe for subjects allergic to caseins.

Monoclonal antibodies are able to identify with high specificity the presence of intact proteins or polypeptidic material conserving antigenic activity. They can be a useful tool to improve quality control of hydrolysed formulae and this control can be specifically aimed at the needs of each highly allergic child. As an example, Mo157 could be used to identify the most suitable formula for a child with a severe allergy to α -casein. Finally, we believe that the development of monoclonal antibodies (for example as biosensors) could improve the quality control of protein hydrolysed products and reduce the risk of anaphylactic reactions in very sensitive subjects.

Acknowledgement

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