

Evaluation by SDS-PAGE and immunoblotting of residual antigenicity in hydrolysed protein formulas

P. RESTANI, T. VELONÀ, A. PLEBANI*, A. G. UGAZIO*, C. POIESI*,
A. MURARO† and C. L. GALLI

*Laboratory of Toxicology, Institute of Pharmacological Sciences, University of Milan, *Dipartimento Materno-infantile e Tecnologie Biomediche, University of Brescia, and †Clinica Pediatrica, University of Padova, Italy*

Summary

Background Extensively hydrolysed protein formulas are widely used as an alternative diet for children with cow's milk allergy. Partially hydrolysed protein formulas have been noted in some studies as useful in the prevention of allergy in infants at high risk of atopy. Although normally well tolerated, these 'hypoallergenic' products have been reported to cause serious immunological reactions in very sensitive subjects.

Objective Starting from these considerations, we studied some commercial hydrolysed formulas in search of biological data supporting the observed clinical reactions.

Methods We set up an electrophoretic method sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which allowed us to study the molecular weight of peptides contained in hydrolysed products. Then, using the immunoblotting technique we evaluated the reactivity of circulating IgE (from serum of children allergic to cow's milk proteins) with the residual intact proteins and with the peptides present in these formulas.

Results Both group of milk proteins (caseins and whey proteins) were important allergens for children included in this study. The presence of high-molecular polypeptides was shown in partial hydrolysed formulas as such and in extensive hydrolysed products after protein enrichment by trichloroacetic acid (TCA) precipitation. Intact residual proteins were mainly responsible for the formation of IgE-antigen complexes observed in immunoblotting. More rarely, polypeptides of partial hydrolysed formulas were involved in immunological responses.

Conclusions Both partial and extensive hydrolysed formulas could induce clinical reactions in very sensitive subjects. These responses are mainly associated with allergy to the small amounts of residual intact proteins.

Keywords: SDS-PAGE, hydrolysed protein formula, alternative diet, cow's milk allergy

Clinical and Experimental Allergy, Vol. 25, pp. 651–658. Submitted 28 September 1994; revised 20 January 1995; accepted 1 February 1995.

Introduction

Cow's milk-based formulas are successfully used for many infants who cannot be breastfed, but special formulas are necessary for those subjects who develop allergy to cow's milk proteins. This pathology is quite

common in the first year of life, although its real incidence is controversial [1–5].

Soy was one of the first alternatives suggested as a protein source in hypoallergenic formulas, but there is evidence that 10–20% of children with cow's milk allergy do not tolerate soy derivatives either [6–9]. The most recent alternative diets are based on hydrolysed protein formulas that have been developed to lower or eliminate

Correspondence: Dr P. Restani, Institute of Pharmacological Sciences, via Balzaretti 9, 20133 Milano, Italy.

the allergenicity of proteins (extensive hydrolysates) or to reduce the antigenic load and the risk of sensitization (partial hydrolysates).

Some clinical studies, performed in children with IgE-mediated cow's milk allergy, showed a crossreactivity between different hydrolysed formulas and cow's milk proteins. This evidence was confirmed by different tests: skin-prick test (SPT), radioallergosorbent test (RAST) and double-blind placebo controlled food challenge (DBPCFC) [10–12]. Cases of anaphylactic reactions to extensively hydrolysed formulas have also been reported [13–15].

Since it has been demonstrated that peptides of 13–17 amino acids are able, when associated with MHC (Major Histocompatibility Complex) class II molecules, to stimulate specific T cell clones [16], we believe that it is not safe or biologically justifiable to base the definition of 'hypoallergenic' only on the low amount of high-molecular weight (mol. wt.) polypeptides present in hydrolysed formulas after the enzymatic treatment.

Starting from these considerations and in search of biological data supporting the clinical reactions to hypoallergenic formulas, we developed a method able to evaluate: the presence of residual cow's milk proteins after industrial hydrolysis; the mol.wt. distribution of oligo and polypeptides present in different commercial formulas (both partially and extensively hydrolysed); the antigenicity of these proteins and/or polypeptidic fragments, using immunoblotting and the serum of cow's milk allergic children.

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_1 -casein (lyophilized powder, chroma-tographically purified; approximately 85% by PAGE); 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); bovine serum albumin (BSA) (essentially globulin free; lyophilized powder; approximately 99% by agarose electrophoresis).

Chemically enriched $\beta + \gamma$ -caseins were prepared by ourselves from a total casein extract [17], following the fractionated precipitation method of Tripathi and Gehrke [18]. Purified proteins were suspended in sample buffer (see SDS-Electrophoresis section) at the final concentration of 0.5–1 mg/mL.

Milk formulas

Samples used in this study included one formula for newborn, containing cow's milk proteins, and six (three partially and three extensively) hydrolysed formulas, with the following characteristics: Vivena 2 (VV, Dieterba, a conventional cow's milk base formula, containing 70% and 30% caseins and whey proteins respectively); Alfaré (AL, Nestlé, an extensive whey hydrolysate); Humana HA (HHA, Humana, partial whey hydrolysate); Nidina HA (NHA, Nestlé, a partial whey hydrolysate); Nutramigen (NT, Mead Johnson, an extensive casein hydrolysate); Pepti Junior (PJ, Nutricia, an extensive whey hydrolysate with short-chain peptides); Pregomin (PR, Milupa, a partial soy-bovine collagen hydrolysate).

Protein precipitation with trichloroacetic acid (TCA)

Both hydrolysed and non-hydrolysed formulas 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' at 4°C, samples were centrifuged (J2-21 Centrifuge, Beckman Instruments, Inc., Palo Alto, CA, USA) for 30' at 4°C and 10 000 rpm. 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 SDS-Electrophoresis section) and loaded onto the electrophoretic gel.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

All chemicals and instruments used in the electrophoretic analyses were purchased from Bio-Rad (Richmond, CA, USA). Before performing the electrophoretic run, samples were suspended in sample buffer (0.25M 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 Neuhoff *et al.* [18], and analysed with a laser densitometer (Ultrosan XL, LKB, Bromma, Sweden). Densitometries were elaborated with the LKB 2400 Gel Scan XL software package. Molecular weight standard solution (Broad range) was purchased from BioRad and contained: rabbit skeletal muscle myosin (mol.wt. = 200 000); *Escherichia coli* β -galactosidase (mol.wt. = 116 250); rabbit muscle phosphorylase b (mol.wt. = 97 400);

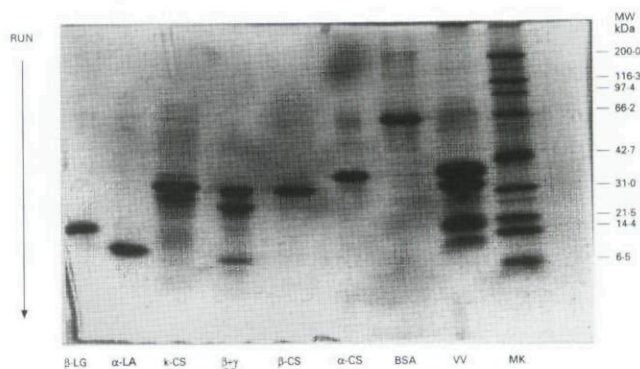
Table 1. Specificity of circulating IgE detected by RAST ($n = 12$)

Milk proteins		Number of subjects ($n = 12$)
α -Lactalbumin	Class IV	2
	Class III	2
	Class II	5
	Class I	2
	Class 0	1
β -Lactoglobulin	Class IV	1
	Class III	1
	Class II	6
	Class I	1
	Class 0	3
Caseins	Class V	3
	Class IV	3
	Class III	6

bovine serum albumin (BSA, mol.wt. = 66 200); hen egg white ovalbumin (mol.wt. = 42 700); bovine carbonic anhydrase (mol.wt. = 31 000); soybean trypsin inhibitor (mol.wt. = 21 500); white hen egg lysozyme (mol.wt. = 14 400); bovine pancreas aprotin (mol.wt. = 6500).

Immunoblotting

After SDS-PAGE, transfer of proteins from the gel onto the polyvinylidene difluoride (PVDF, Immobilon P; Millipore, Bedford, MA, USA) membrane is achieved by electrophoretic elution (wet transfer). The transfer buffer was: Tris 25 mM, glycine 193 mM and methanol 20%. To verify the protein transfer, 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% and washed three times with 0.25% gelatin (Bio Rad) solution (in 150 mM NaCl, 5mM EDTA, 50 mM Tris, 0.05% TRITON-X) to prevent non-specific adsorption of the immunological reagents. Membrane was then immersed in 10 ml of 0.25% gelatin solution, containing 0.3 mL of serum from allergic children. Antigen-IgE complex was detected using anti-human IgE antibodies labeled 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).

**Fig. 1.** - SDS-PAGE of milk proteins. CS. casein. α -LA. α -lactalbumin. β -LG. β -lactoglobulin. BSA. bovine serum albumin. VV. Vivena 2 MK. MW standard solution.

Clinical data

Serum samples were from atopic children (seven males and five females, age ranging from 5 months to 6 years, mean age 2.3 years), with RAST positivity to cow's milk proteins; the class of RAST positivity to different cow milk proteins is shown in Table 1.

As a negative control, sera of 10 children allergic to foods other than milk and four non-allergic adult subjects were used.

Results

SDS-PAGE

In order to obtain both a good separation of milk proteins and the analysis of a wide molecular weight range, we set up an electrophoretic method based on a gradient running gel. Starting from a previously published method [20], the composition of electrophoretic gels were modified as follows: *gradient running gel*: 9–19% 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'-tetramethylenediamine (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.

Milk protein separation

The electrophoretic distribution of milk proteins in the 9–19% gradient-gel is shown in Fig. 1.

The different caseins are well separated although they do not always migrate in relation to their mol. wt., as

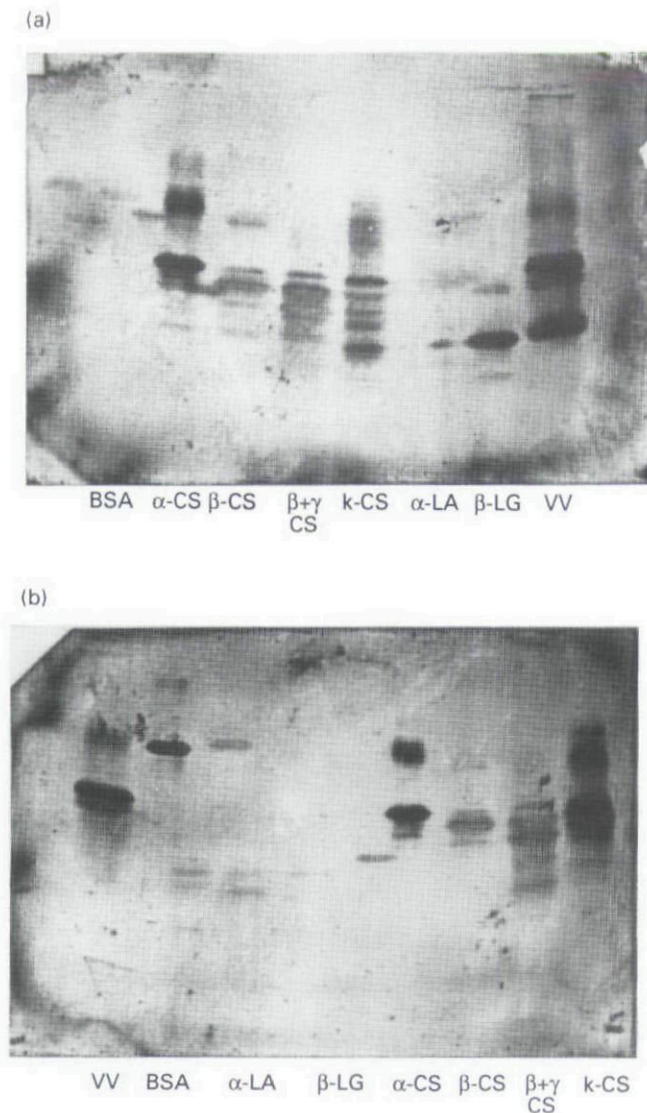


Fig. 2. Immunoblotting of purified milk proteins. The serum used was from two children allergic to cow's milk (abbreviations as in Fig. 1).

expected in a SDS-PAGE. In fact, in spite of their very close mol.wts., α_s (23 600) and β (24 000) caseins migrate differently in the gel. The other caseins and whey proteins (α -lactalbumin, β -lactoglobulin and BSA) regularly run according to their molecular weights. In $\beta + \gamma$ -casein sample, three major bands are visible: from the gel start they correspond to β , γ_1 and γ_{2-3} caseins. The κ -casein shows some heterogenic fractions.

Reactivity of circulating IgE against purified cow's milk proteins in immunoblotting

Immunoblottings of two of the 12 children included in

Table 2. Specificity of circulating IgE detected by immunoblotting

Milk proteins	Number of positive responses (n = 12)
Caseins	12
α -casein	12
β -casein	8
γ -caseins	7
κ -casein	11
Whey proteins	11
α -lactalbumin	6
β -lactoglobulin	5
BSA	5

this study are shown in Fig. 2. Child 1 reacts strongly to α -casein, κ -casein and β -lactoglobulin, and moderately to β and γ caseins; child 2 is highly positive to α -casein, κ -casein and BSA, mild response was observed for β - and γ -caseins. In the milk sample (Vivena 2 [conventional milk base formula]) the reactivity to the different allergens is clearly visible. Immunological responses of the 12 children included in the study are reported in Table 2.

Results are in agreement with RAST positivity; in fact caseins seem to be the most important milk allergens for these children. Comparable responses were also obtained with whey proteins. The specificity of binding to the different transferred fractions was demonstrated performing the immunoblotting in the same experimental conditions and using the sera of 10 allergic children (allergic to foods other than milk) and of four adult non-allergic subjects (of our laboratory). No positive response was observed.

Thanks to the high specificity of the test, immunoblotting permits an easy identification of responses to different milk proteins. In particular, α and κ -caseins are the strongest allergens. Moreover, BSA (not tested in RAST) represents an important allergen for five of the children included in this study.

SDS-PAGE of hydrolysed formulas

When 'hypoallergenic-hypoantigenic' samples as such are loaded on SDS-PAGE gel, the presence of protein bands is observed only in full milk (Vivena 2) and partially hydrolysed formulas, that is Humana HA, Nidina HA (partial whey hydrolysates) and Pregomin (partial soy-collagen hydrolysate) (gel not shown). After enriching of the protein fraction by TCA precipitation, each milk formula is visible in SDS-PAGE with a characteristic pattern (Fig. 3). As expected, partial hydrolysed formulas present the higher amount of

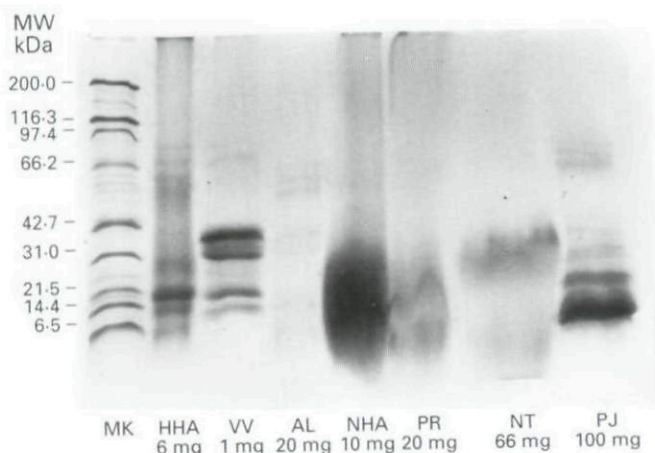


Fig. 3. SDS-PAGE of hydrolysed formulas after protein enrichment by TCA precipitation (abbreviations as in Materials and Methods section). The quantities (mg of formula as such, before of TCA precipitation) loaded onto the gel are reported.

polypeptides, while extensively hydrolysed products (Alfare; Nutramiger and Pepti Junior) show a lower residue, often concentrated in only a few proteic bands. The polypeptides present in the analysed formulas show the following molecular weight distribution:

- HHA a diffused area ranging from 71 000 and 6000 Da, with two important bands corresponding to whey proteins;
- NHA a diffused area ranging from 35 000 to < 6500 Da (approximately 2500 Da), without well defined bands;
- PR a diffused area between 20 000 and < 6500 Da (approximately 3000 Da), with a band of precipitation close to 14 000 Da;
- AL a low quantity of protein associated to six major bands; three of them corresponding to α , β -casein and α -lactalbumin
- NT a diffused area with two major bands having mol.wt. = 18 000 and 5500 Da;
- PJ several well defined bands concentrated in the area of whey proteins; the most important band corresponds to α -lactalbumin.

Reactivity of circulating IgE against TCA-precipitated formulas in immunoblotting

When immunoblotting was performed after the SDS-PAGE of TCA-precipitated formulas, a well defined IgE-antigen complex was often observed for both partial and extensive hydrolysed samples. Figure 4 illustrates the response obtained with the serum of two children previously used for the immunoblotting with purified

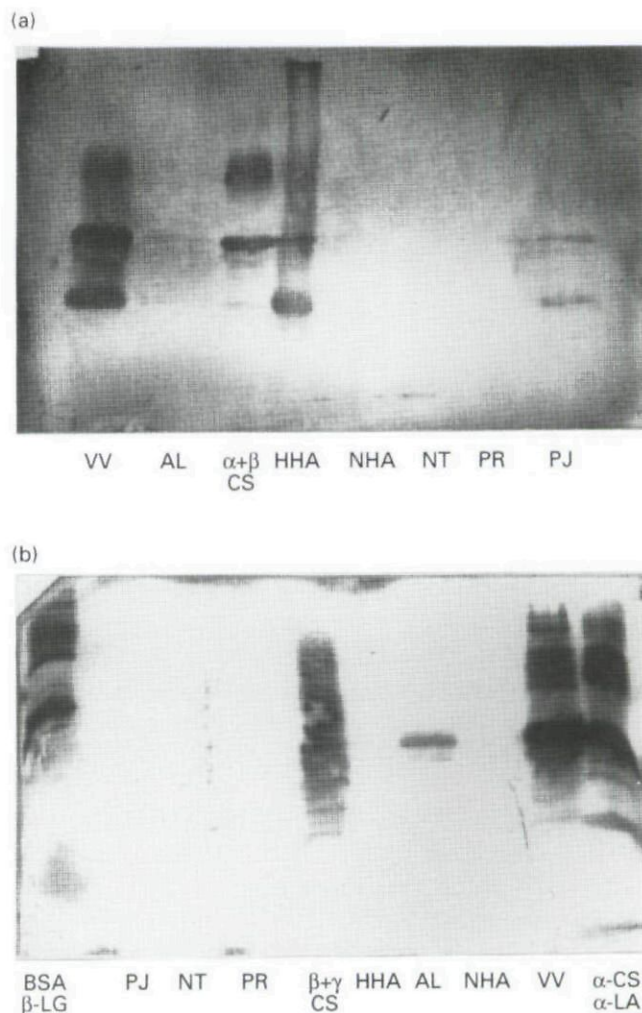


Fig. 4. Immunoblotting of hydrolysed formulas. The serum used was from the same children of immunoblottings shown in Fig. 2

cow's milk proteins (Fig. 2). In this membrane, responses to Alfaré (mainly α -casein), Humana HA (α -casein and β -lactoglobulin) and Pepti Junior (α -casein and β -lactoglobulin) are clear for child 1. Child 2 reacts mainly to Alfaré (α -casein).

The responses of the 12 children included in our study are shown in Table 3. No positive response was found when non-specific binding was investigated. Generally the most severe responses involve the residual intact milk proteins, but in some cases (especially in partial hydrolysates HHA and NHA) IgE complexes with polypeptidic fragments are also detectable.

DISCUSSION

Usually electrophoretic separation of caseins is performed

Table 3. Positive responses to hydrolysed formulas obtained by immunoblotting

Milk formula	Protein source	Positive responses (n = 12)
<i>Milk protein formula</i>		
Vivena 2	Full milk	12
<i>Partial hydrolysed formulas</i>		
Humana HA	Whey	5
Nidina HA	Whey	4
Pregomin	Soy-collagen	1
<i>Extensive hydrolysed formulas</i>		
Alfaré	Whey	8
Nutramigen	Casein	3
Pepti Junior	Whey	4

by UREA-PAGE [21], where caseins move according to their specific ionic charge, and caseins are named α , β and γ in order of their decreasing electrophoretic mobility.

Since the aim of our study was to analyse the molecular weight distribution in milk powder and in hydrolysed protein formulas (hypoallergenic preparations), we could not use UREA-PAGE; in fact this technique does not allow the separation of proteins on the basis of their molecular weights. To reach both the resolution of milk proteins and the analysis of a wide range of molecular weight, we developed an SDS-PAGE, where the run was performed in a gradient of 9–19% acrylamide.

In these electrophoretic conditions, all milk proteins are well separated and in general their mobility is inversely proportional to the molecular weights, as expected in the SDS-PAGE. The only exceptions to this rule are α_s and β -caseins, which present different mobilities in spite of their very close molecular weights. This behaviour could be explained by the presence of a different number of phosphorylated groups (8 vs 5, respectively), which modify the binding of SDS to the two proteins. On the other hand, this anomaly is very useful to us, since in the usual conditions, α_s and β -caseins could not be separated. Because of their very close molecular weights, only caseins γ_2 and γ_3 could not be separated by this technique; finally the observed heterogeneity of κ -casein, due to the carbohydrate moiety, is in agreement with previous studies [22, 23].

By immunoblotting, IgE-antigen complexes are clearly identifiable and the responses illustrated in Fig. 2 and Table 2 prove the high complexity associated with the

milk allergy. In fact both groups of milk proteins (caseins and whey proteins) are important allergens for all of the 12 children included in this study; in particular, the most important allergens are α -casein (12/12 positive responses) and κ -casein (11/12 responses). The high allergenicity of κ -casein, which has not been previously reported, could be associated with its glycoproteic nature as shown for other food allergens [24]. The frequency of positivity for casein in RAST and immunoblotting is similar (12/12), while positivity for α -lactalbumin and β -lactoglobulin was respectively present in 11/12 and 9/12 children by RAST and in 6/12 and 5/12 by immunoblotting. This difference may be due to the structure modification of proteins after SDS-treatment and to the fact that only strong responses were considered positive in immunoblotting.

The hydrolysed formulas, which are widely used as a preventive or therapeutic diet for allergic children, have often been criticized because of the clinical symptomatology associated with their intake. These symptoms could be caused by the residual presence of intact proteins or by the polypeptidic fragments, coming from the hydrolytic processes, which could maintain the antigenic activity. The presence of high-molecular polypeptides is easily identifiable in partial hydrolysed products (as expected), while to obtain the same result in extensive hydrolysed products it is necessary to enrich the protein fraction by TCA precipitation. By this procedure, all formulas show the presence of residual intact proteins with or without other high-molecular weight hydrolytic products. In our conditions, intact residual proteins are mainly responsible for the

formation of IgE-antigen complexes. More rarely, polypeptides of partial hydrolysed formulas are involved in immunological responses. The least immunogenic formula in our study seems to be pregomin; this fact correlates well with the composition of this specific formula which is obtained by the hydrolysis of soya proteins and collagen. The only positive response is probably due to a polyallergy of the child who reacts to soya and/or to collagen.

Considering the quantity of milk formula necessary to show protein residue in SDS-PAGE (from 6 to 100 mg) (see Fig. 3) and the quantity of formula consumed daily by a child (5-6 meals $\times$ 13-15 g/each) it is possible to formulate two conclusions: the presence of significant amounts of intact proteins in partial hydrolysed formula (especially Humana HA) makes their role highly questionable in allergy prevention; and although the quantity of intact proteins present in extensive hydrolysed formulas is tolerated by most children, it could be enough to stimulate immunological responses in very sensitive subjects. For this reason, a specific allergen identification by SDS-PAGE and immunoblotting could represent a useful screening test when a new milk formula must be introduced in the diet of allergic subjects.

It seems important to underline that IgE-producing plasma cells of allergic children are able to identify caseins in whey-protein-hydrolysed formula (i.e. α -casein in Alfaré) and vice versa, showing that the technological separation of caseins from the whey proteins may not always be satisfactory.

In conclusion, the results here reported could contribute to a better comprehension of the biological bases of milk allergy. On the other hand, an improved knowledge of specific antigenic determinants is, in our opinion, an essential step to explain clinical reactions and formulate new-alternative-well-tolerated diets. In fact, since allergenic potential seems to be associated almost exclusively to the residual intact proteins, we could obtain the production of formulas where only allergenic sequences are eliminated. As a consequence, the nutritional value, tolerogenic activity and organoleptic characteristics could be improved by a reduced hydrolysis of proteins. Finally, considering the significant difference between human and cow's milk proteins, the use of a protein source other than bovine milk could be justifiable.

Acknowledgements

We thank Mrs Helen Downes for editorial assistance.

References

- 1 Working Group on Cow's Milk Allergy — Italian Working

- Group for Pediatric Gastroenterology of the Italian Society of Pediatrics. Cow's milk allergy in the first year of life. An Italian collaborative study. *Acta Paed Scand* 1988; 348 (suppl.): 3-14.
- 2 Bahna SL. Milk allergy in infancy. *Ann Allergy* 1987; 60: 131-6.
- 3 Wilson NW, Hamburger RN. Allergy to cow's milk in the first year of life and its prevention. *Ann Allergy* 1988; 61:323-328.
- 4 Lebenthal E. Cow's milk protein allergy. *Pediatr Clin North Am* 1975; 22:827-33.
- 5 Lindfors A, Enocksson E. Development of atopic disease after early administration of cow milk formula. *Allergy* 1988; 43:11-6.
- 6 Businco L, Bruno G, Giampietro PG, Cantani A. Allergenicity and nutritional adequacy of soy protein formulas. *J Pediatr* 1992; 121:S21-28.
- 7 Ament ME, Rubin CE. Soy-protein — another cause of the flat intestinal lesion. *Gastroenterology* 1972; 62: 227-34.
- 8 Whittington PF, Gibson R. Soy protein intolerance: four patients with concomitant cow's milk intolerance. *Pediatrics* 1977; 59:730.
- 9 Perkkio M, Savilahti E, Kuitunen P. Morphometric and immunohistochemical study of jejunal biopsies from children with intestinal soy allergy. *Eur J Pediatr* 1981; 137:63-9.
- 10 Rugo E, Wahl R, Wahn U. How allergenic are hypoallergenic infant formulae? *Clin Exp Allergy* 1992; 22:635-9.
- 11 Ragno V, Giampietro PG, Bruno L, Businco L. Allergenicity of milk protein hydrolysate formulae in children with cow's milk allergy. *Eur J Pediatr* 1993; 152:760-2.
- 12 Kleinman RE. Cow milk allergy in infancy and hypoallergenic formulas. *J Pediatr* 1992; 121:S116-21.
- 13 Businco L, Cantani A, Longhi A, Giampietro PG. Anaphylactic reactions to a cow's milk whey protein hydrolysate (Alfa-Ré, Nestlé) in infants with cow's milk allergy. *Ann Allergy* 1989; 62:333-5.
- 14 Saylor JD, Bahna SL. Anaphylaxis to casein hydrolysate formula. *J Pediatr* 1991; 118:71-4.
- 15 Sampson HA, James JM, Bernhisel-Broadbent J. Safety of an amino acid-derived infant formula in children allergic to cow milk. *Pediatrics* 1992; 90:463-5.
- 16 Rudensky AY, Preston-Hurlburt P, Hong S-C, Barlow A, Janeway Jr. CA. Sequence analysis of peptides bound to MHC class II molecules. *Nature* 1991; 353:622-7.
- 17 Resmini P, Saracchi S, Miotti G. L'aldeide formica nel formaggio grana padano. I. Sua distribuzione nei prodotti della caseificazione e nel formaggio in maturazione. *L'industria del latte* 1980; 16:3-16.
- 18 Tripathi KK, Gehrke CW. Chromatography and characterization of gamma casein. *J Chromatography* 1969; 43:322-31.
- 19 Neuhoff V, Arold N, Taube D, Ehrhardt W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 1988; 9:255-62.
- 20 Sampson HA, Bernhisel-Broadbent J. Yang E., Scanlon

- SM. Safety of casein hydrolysate formula in children with cow milk allergy. *J Pediatrics* 1991; 118:520–5.
- 21 Restani P, Campagner P, Fiecchi A, Resmini P, Galli CL. Identification of spinacine as the principal reaction product of γ -casein with formaldehyde in cheese. *Fd Chem Toxic* 1988; 26:441–6.
- 22 Doi H, Ibuki F, Kanamori M. Heterogeneity of reduced bovine κ -casein. *J Dairy Sci* 1979; 62:195–203.
- 23 Doi H, Park BS, Ibuki F, Kanamori M. Heterogeneity and composition of κ -casein from bovine colostrum. *Agric Biol Chem* 1980; 44:813–20.
- 24 American Academy of Allergy and Immunology. Adverse reactions to food. Cap. 2. Chemistry of selected food antigens. NIH Publication No. 84.2442, 1984.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.