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Characterization of antigens and allergens in hypo-allergenic infant formulae

Received: 15 April 1993
Accepted: 13 October 1994

Abstract The antigenicity and allergenicity of so-called hypo-allergenic infant formulae is mainly determined by the degree of hydrolysis and ultra-filtration. Five different formulae were investigated by means of immunoblotting and RAST in order to characterize the antigens and allergens regarding their molecular weights, molecular origin and their ability to bind human IgG and IgE antibodies: A non hydrolysed infant formula (I-F), a mixture of the major cow's milk proteins (PM), a whey-based infant formula (W-H), a whey-based and ultra-filtrated infant formula (U-H), a casein/whey-based infant formula (CW-H). By immunoblotting we demonstrated that all tested formulae still contain antigens with molecular weights from 3 to 67 kD. But when compared with I-F and PM the antigen content of the hydrolysed formulae was considerably lower. The lowest antigen content could be demonstrated in U-H, which contains casein fragments (3–6 kD) and beta-lactoglobulin and its fragments (6–18 kD). W-H and CW-H contain bovine serum albumin, beta-

lactoglobulin, casein and their fragments (3–67 kD). All hydrolysed formulae tested showed a reduced IgE-binding capacity. Three out of 12 cow's milk allergic children possessed IgE binding to U-H or W-H, and 5 of them IgE against CW-H.

Conclusion The enzymatic hydrolysis plus ultra-filtration seems to be the most efficient method to reduce the antigen content of so-called hypo-allergenic infant formulae.

Key words Hypo-allergenic infant formula · Allergen · Antigen · Immunoblotting · RAST

Abbreviations ALA alpha-lactalbumin · BLG beta-lactoglobulin · BSA bovine serum albumin · CMA cow's milk allergic · CW-H casein-whey-based hypo-allergenic infant formula · I-F non-hydrolysed whey-based infant formula · PBS phosphate-buffered saline · PM protein mixture · RAST radio-allergo-sorbent test · U-H whey-based ultra-filtrated hypo-allergenic infant formula · W-H whey-based hypo-allergenic infant formula

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Introduction

Characterization of antigens and allergens in hypo-allergenic infant formulae

Food allergies are among the first manifestations of atopy and are thought to be gate openers for other atopic dis-

eases [5, 8, 9, 13, 42, 47]. In adults a multiplicity of vegetables, spices, fish, eggs, cow's milk protein and fruits cause allergies [45], whereas the leading cause of food allergy in infants is cow's milk protein [5]. Between 0.3% [22] and 7.5% [13] of all infants suffer from cow's milk allergy resulting from the use of cow's milk as the main source of protein. Soyprotein based infant formulae con-

tain intact soyproteins which have nearly the same rate of sensitization as cow's milk proteins [3, 8, 15, 25]. Thus, generally breast-feeding still remains the best form of nutrition during the first 6 months of life [4, 6, 18, 22, 32, 46, 47].

Hypo-allergenic infant formulae – mostly based on hydrolysed cow's milk proteins – show only a residual antigenic/allergenic activity, after most of the epitopes are enzymatically digested. This procedure leads to a reduced but still existing IgG- and IgE-binding capacity [1, 2, 6, 11, 26, 41]. Although the use of such products aims to prevent allergy [4, 8, 9, 15, 19, 43, 47], few of them are suited for the nutrition of cow's milk allergic (CMA) patients [7, 23, 34]. Such hydrolysates are nutritionally sufficient, but a high load of unprocessed protein may be important for the induction of immune tolerance [12, 20], which in a strict sense still has to be proven for IgE in humans. However, as far as therapy is concerned the content of allergens and antigens should be as low as possible. Consequently it seems to be of great importance to obtain more detailed information about the immunoreactivity of the proteins and peptides in hypo-allergenic infant formulae. The aim of this study is to characterize the antigens and allergens in hypo-allergenic infant formula by means of immunoblotting and radio-allergo-sorbent test (RAST).

Methods

Patients and determination of the IgG- and IgE-binding capacity

All subjects were 1–5 years old and characterized as follows: healthy subjects must have a negative history of atopy and an IgE serum concentration within the normal range, whereas atopic children demonstrated an elevated serum concentration of IgE and a positive skin prick test, but not with cow's milk allergy. CMA subjects showed a clear type I allergic reaction on challenge, were positive in skin prick test and had serum IgE antibodies against cow's milk as measured by RAST (Pharmacia, Freiburg, Germany).

The IgG-binding capacity was investigated by sodium-dodecylsulphate electrophoresis with succeeding immunoblotting. Sera of 7 healthy, 7 atopic and 7 CMA children were tested.

The IgE-binding capacity was investigated by means of RAST (Pharmacia). The antigens (hypo-allergenic infant formulae) were kindly coupled on paper discs by Pharmacia (Uppsala, Sweden). We tested sera of 12 healthy, 12 CMA and 8 atopic subjects, who were classified as described.

Substances

The following substances were investigated: whey-based formula (W-H; Beba HA), whey-based ultra-filtrated formula (U-H), casein-whey-based formula (CW-H; Aptamil hypo-antigen). A non-hydrolysed whey-based formula (I-F; Bebal) and a mixture (PM) of the nonhydrolysed major cow's milk proteins alpha-lactalbumin (ALA), β -lactoglobulin (BLG), bovine serum albumin (BSA) and casein served as control material.

Electrophoresis

Polyacrylamide gel electrophoresis (15%–28% T, 0.4% C) was performed as described by Schagger and Jagow [36]. Protein of the hydrolysed formulae (100 mg) and 20 mg of the non-hydrolysed material, respectively, were dissolved in 1 ml Laemmli sample buffer [24]. The PM contained 1 mg casein, 1 mg BSA, 2 mg ALA and 2 mg BLG/ml sample buffer.

The samples were put into an ultrasonic bath (50 W) for 5 min and centrifuged for 5 min at 10 000 g. The supernatant was heated at 95°C for 5 min and used for electrophoresis. We used protein markers of 3–67 kDa (Sigma, Munich, Germany).

Immunoblotting

After electrophoresis the proteins were transferred to an immobilone membrane (Millipore, Eschborn, Germany) using a blotting apparatus (Multiphor-2 and Multiphor-2-Nova-Blotkit, Pharmacia). In order to block the free binding sites on the immobilone membrane, it was incubated for 3 × 5 min in a solution of phosphate-buffered saline (PBS)-Tween (180 mM NaCl, 100 mM Na₂HPO₄ 2 H₂O, 0.05% Tween, pH 7.2). After this the immobilone membrane was incubated overnight at 4°C with hyperimmune rabbit antisera against ALA, BLG, casein and BSA (Drs. Jost and Monti, Nestlé-Research-Centre, Vevey, Switzerland). Some of these antisera proved to contain antibodies against different cow's milk proteins. We tried to eliminate the non-specific antibodies by absorbing them with cow's milk proteins for 1 h at 37°C. The rabbit antisera were diluted in PBS-Tween (1:1000) and to 1 ml of the dilution the following proteins were added: the anti-BLG-serum 1 mg casein and 1 mg ALA; to the anti-ALA serum 1 mg BSA and 1 mg BLG, and to the anti-casein-serum 1 mg BSA and 1 mg BLG. No preabsorption was necessary for the anti-BSA serum because we did not see any reaction of this serum with proteins other than BSA (data not shown). From the immobilone membrane the first antisera were washed off with PBS-Tween for 2 × 15 min. The biotinylated antirabbit antibody (Sigma, Munich, Germany) was added for 1 h at room temperature, diluted in PBS-Tween (1:10 000). After another washing the membrane was incubated for 2 h at room temperature with a 1:1000 diluted avidin-peroxidase-conjugate (Sigma, Munich, Germany) and stained using 4-chloro-1-naphthol (Sigma, Munich, Germany) and H₂O₂ (Merck, Darmstadt, Germany).

The binding-capacity of IgG-antibodies from human sera was determined by immunoblotting with individual sera of healthy, atopic and CMA persons as well as three pooled sera of each group. The immobilone membrane was incubated overnight at 4°C with human sera (diluted 1:80 in Tween-PBS). After washing 3 × 15 min with PBS-Tween the membrane was incubated for 3 h with peroxidase-conjugated antihuman IgG (Dakopatts, Hamburg, Germany) diluted in PBS-Tween (1:200). 4-Chloro-1-naphthol and H₂O₂ were used for staining.

Results

Hypo-allergenic infant formulae: remaining antigens and their molecular weights

A specific binding of rabbit anti-ALA serum with I-F, PM and W-H was demonstrated by immunoblotting (Table 1). Only the PM showed a positive reaction after preincubation of the anti-ALA serum with BLG and BSA. The formulae W-H and CW-H as well as PM and I-F reacted with the anti-BLG serum (Table 1). Preincubating this serum with

Table 1 Molecular origin of proteins and peptides in hypo-allergenic infant formulae (U-H, W-H, CW-H; I-F and PM served as controls), investigated by sodium dodecylsulphate-electrophoresis and immunoblotting with hyperimmune rabbit antisera against single cow's milk proteins. Results before and after absorption of antisera with those cow's milk proteins, they should not bind to

Formula blotted	Antisera against			
	ALA	BLG	BSA	Casein
<i>Original sera</i>				
U-H	—	+	—	+
W-H	+	+	+	+
CW-H	—	+	+	+
I-F	+	+	+	+
PM	+	+	+	+
<i>After absorption</i>				
U-H	—	—	ND	+
W-H	—	—	ND	+
CW-H	—	—	ND	+
I-F	—	—	ND	+
PM	+	+	ND	+

casein and ALA resulted in a diminished binding: only the PM still gave a positive reaction. This was interpreted as a significant absorption of the unspecific antibodies by the proteins added. Therefore in the following tests both the original and the absorbed rabbit antisera were used.

BSA and its fragments could be demonstrated in PM, I-F, W-H, and CW-H, but not in U-H (Table 1). The I-F, the PM and all hydrolysates showed a specific binding of the casein antiserum. The molecular weights of the visualized antigens ranged between 3 and 67 kDa (not shown). In the case of U-H only casein derived peptides of 3–6 kDa could be demonstrated (Table 1, Fig. 1b).

Antigens in hypo-allergenic formula and their ability to bind human IgG-antibodies

As expected the PM as well as I-F bound human IgG-antibodies from the sera of healthy, atopic and CMA sub-

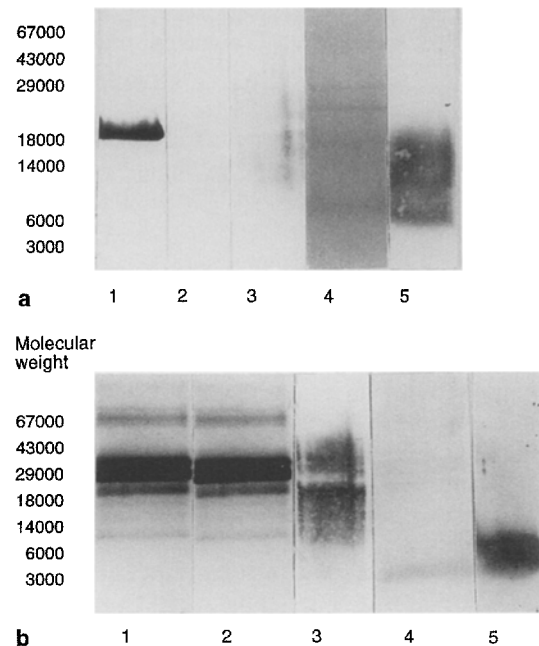


Fig. 1 Binding of hyperimmune rabbit antisera against BLG (a) and casein (b), respectively, to antigens of hypo-allergenic infant formula: 1 Protein mixture containing ALA, BLG, casein, BSA (PM); 2 I-F; 3 CW-H; 4 W-H; 5 U-H

jects. The visualized proteins were BLG (18 kDa), casein (29 kDa) and BSA (67 kDa). ALA (14 kDa) could only be demonstrated with the pooled sera of CMA subjects (Fig. 2).

Two hydrolysate formulae (W-H, CW-H) showed binding of IgG antibodies from pooled human sera. These antigenic molecules had molecular weights between 6 and 67 kDa. Two individual sera of healthy subjects contained IgG antibodies binding to the 3–6 kDa fragments in U-H (not shown).

IgE-binding-capacity of hypo-allergenic formulae

Neither the hydrolysates nor the I-F bound IgE-antibodies from the sera of the 12 healthy persons tested. In one out

Fig. 2 Binding IgG antibodies of pooled human sera from healthy, atopic and CMA children to antigens of hypo-allergenic infant formula, demonstrated by immunoblotting: 1 Protein mixture containing ALA, BLG, casein, BSA (PM); 2 I-F; 3 CW-H; 4 W-H; 5 U-H

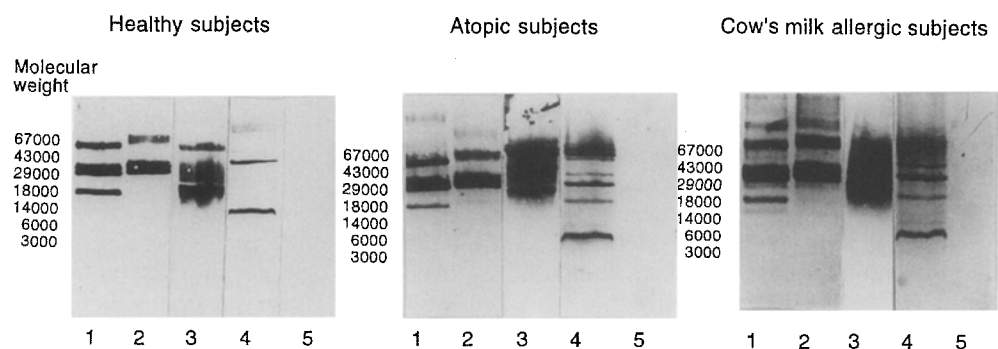


Table 2 Frequency of RAST classes of the specific IgE antibodies in sera of 12 CMA infants, against I-F and the following hypo-allergenic infant formulae: U-H, W-H, CW-H

Formula	Specific IgE (RAST class)				
	0	1	2	3	4
U-H	9	1	2	—	—
W-H	9	1	2	—	—
CW-H	7	—	5	—	—
I-F	—	1	3	6	2

of eight atopic individuals we could demonstrate IgE (RAST class 1) against U-H, W-H and CW-H, IgE binding to I-F was not seen (data not tabulated). In contrast to these results all 12 CMA children had IgE antibodies against the non-hydrolysed proteins. Five of these patients showed IgE antibodies against CW-H, three of them exhibited IgE antibodies against U-H and W-H (Table 2).

Discussion

Several studies have demonstrated an IgG- and IgE-binding capacity of hypo-allergenic infant formulae [1, 25, 28, 29, 31, 38, 40, 44], which in some cases caused anaphylactic reactions [2, 6, 11, 17, 18, 33, 35]. We tried to determine molecular weights and the molecular origin of the antigens and allergens present in hypo-allergenic infant formulae. Immunoblot investigation demonstrated a slightly positive reaction of W-H infant formula with rabbit ALA-antiserum (not shown). Even after absorption of the serum with BSA and BLG the formula was shown to possess reactive antigens with molecular weights of 6 and 29 kDa. Before absorption of the BLA-antiserum with casein and ALA, all hypo-allergenic formulae showed a positive reaction in the immunoblot, whereas the absorbed serum detected proteins only in the PM (18 and 29 kDa). In the hypo-allergenic infant formulae and in the non-hydrolysed I-F no intact BLG could be shown. This effect can be explained by a contamination of the casein and ALA with BLG, absorbing a considerable amount of the antibodies against BLG. Thus, the hydrolysates tested contain at least BLG fragments with a molecular weight of about 6 kDa.

The immunoblot with rabbit hyperimmune casein antiserum showed the W-H infant formula and the CW-H formula to contain remaining proteins and peptides with a molecular weight of 6–43 kDa, and U-H formula peptides of 3–6 kDa, respectively. Thus, even the whey-based hypo-allergenic infant formulae contain casein and its fragments. This observation strongly suggests a contamination of whey proteins with caseins or vice versa [37, 39]. Another explanation might be the contamination of vitamin or mineral supplements with casein. Rabbit hyperimmune antisera against whey proteins did not react with casein.

The casein antiserum reacted with an 18 kDa protein, possibly BLG, but this finding persisted after absorption of the anti-casein serum with BLG. Thus, this protein contains either casein epitopes or is BLG. This last assumption is based on the fact that, so far, all anti-casein antibodies are raised by immunising animals with purified caseins which inevitably contain traces of BLG. Thus, these antisera may contain specific BLG antibodies and it might be questioned whether the absorption has completely removed these antibodies. Intact BSA could be demonstrated in the W-H and in the CW-H but not in the U-H hypo-allergenic infant formulae (Table 2). This stresses the possibility of reducing the antigen-content by ultra-filtration.

Immunoblot investigations with human sera from healthy, atopic and CMA infants showed that proteins and peptides in hypo-allergenic formulae are able to bind human IgG antibodies. These antigens have molecular weights of 3–67 kDa. The 3–6 kDa fragments existing in the U-H infant formula are able to bind IgG antibodies of two healthy children, a binding with the IgG antibodies out of the pooled sera of healthy, atopic and CMA infants was not seen, apparently due to dilution by pooling. Oldaeus et al. [27] could show a weak binding of human IgE antibodies to a 5 kDa peptide deriving from a new ultra-filtrated whey-based infant formula.

From our results of immunoblotting one can conclude that ultra-filtration following an enzymatic hydrolysis is the most efficient process to reduce the antigen content in milk. Correspondingly, Jost et al. [21] could not demonstrate a sensitisation of rabbits by this formula. In a mouse-model Poulsen and Hau [30] showed that peptides with a molecular weight above 6500 lead to passive cutaneous anaphylaxis, whereas peptides with a molecular weight between 3400–6500, and peptides <3400 provoked no reaction. On the other hand Seban et al. [38] demonstrated the production of specific IgG antibodies against a casein-based nonultrafiltrated formula. Regarding the IgE binding the RAST could not show any difference between U-H and W-H. This had been shown by others [33, 44] and may result from the fact that both formulae had been coated in excess on a cyanbromide-activated cellulose membrane, e.g. even from the U-H a sufficient number of epitopes may be present to bind IgE antibodies.

In conclusion: by means of immunoblotting and RAST a significant reduction in antigen and allergen content can be documented for all three hypo-allergenic preparations tested. The antigens demonstrated were derived from all major cow's milk proteins. Even in the whey-based formulae casein and its fragments could be demonstrated. One can conclude that the reduction of the variability of protein sources in hypo-allergenic infant formulae does not seem to be advantageous, because there are still some caseins in whey proteins. Therefore one should be careful in administering hypo-allergenic infant formulae to cow's milk allergic patients.

Concerning the prevention of atopic diseases we would like to refer to a position paper of ESPACI stating the following [7, 10]: "Only extensively hydrolyzed cow's milk formulae have been shown to protect children from sensitisation to cow's milk allergy and early manifestations of atopy. This beneficial effect appears to be limited to re-

duced risk of dermal and gastro-intestinal allergic symptoms in the first years of life."

Acknowledgements The technical advices regarding the sodium-dodecylsulphate-electrophoresis from Dr. I. Krause (Weihenstephan, Germany) is gratefully acknowledged. The study was supported by Milupa AG (Germany) and Nestlé AG (Switzerland).

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