

Determination of allergenicity to three cow's milk hydrolysates and an amino acid-derived formula in children with cow's milk allergy

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Summary

Background Products based on hydrolysed cow milk proteins or amino acid mixtures are recommended in children with cow's milk hypersensitivity. However, some children who are allergic to cow's milk and who clinically react to substitute milk formulas have been observed.

Objective To determine the tolerance and allergenicity of protein hydrolysate or amino acid-derived formulas in children with IgE-mediated cow's milk allergy.

Methods Twenty children with positive cow's milk challenges, positive skin prick tests and/or serum-specific IgE antibodies to cow's milk were selected. Oral challenges, skin prick tests and serum-specific IgE antibodies to extensively hydrolysed whey formula, partially hydrolysed whey formula, extensively hydrolysed casein formula and amino acid-derived formula were performed.

Results Five out of 17 (5/17) children reacted to partially hydrolysed whey formula, (3/16) to extensively hydrolysed whey formula, (2/10) to amino acid-derived formula, (1/16) to extensively hydrolysed casein formula. Only extensively hydrolysed casein formula was tolerated by at least 90% (with 95% confidence intervals) of children. Hydrolysates provoked early and delayed clinical reactions, amino acid mixtures only delayed reactions. Partially hydrolysed whey formula elicited a significantly higher number of positive skin prick test reactions than other formulas. Two children had specific IgE antibodies to extensively hydrolysed whey formula, one to partially hydrolysed whey formula, one to extensively hydrolysed casein formula and none to amino acid-derived formula.

Conclusion In this study, none of the cow's milk substitutes has been found to be non-allergenic. Our results suggest that in children with IgE-mediated cow's milk allergy, the first ingestions of extensively hydrolysed cow's milk protein formulas require strict medical supervision because of immediate reactions. This is not the case for amino acid-derived formula. Moreover, our data suggest that treatment of children allergic to cow's milk with cow's milk substitutes should be monitored for several days to document tolerance.

Keywords cow's milk allergy, children, hydrolysed protein formulas, amino acid-based formula

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Introduction

The treatment of cow's milk allergy (CMA) consists of avoiding cow's milk (CM) proteins. The use of soy-based formula as a milk substitute, is controversial. According to a joint statement of the Committee on Hypoallergenic Formulas of the European Society for Paediatric Allergology and Clinical Immunology and of the Committee on Nutrition of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition [1], formulas based on soy protein are not useful for the initial treatment of food allergy since soy hypersensitivity occurs in a number of children with CMA [2]. On the other hand, soy allergy has been documented in only 14% of children with IgE-mediated CMA [3]. Consistently the Nutrition Committee

of the American Academy of Pediatrics has stated that most infants with IgE-mediated CMA do well on soy formulas [4].

Among hydrolysed CM protein hydrolysates, formulas with moderately reduced allergenicity, based on partially hydrolysed CM proteins can frequently induce adverse reactions, even anaphylactic [5], and are not recommended for the treatment of milk-sensitive children [1].

Products with highly reduced allergenicity based on extensively hydrolysed CM proteins or amino acid-based formulas are recommended in children with CM hypersensitivity [1]. However, cases of anaphylactic reactions to both extensively whey hydrolysates [6] and extensively casein hydrolysates [7,8] have been sparsely reported in children with CMA. Few trials have studied by double-blind controlled challenge tests the clinical tolerance of high degree hydrolysed CM formulas in children with IgE-mediated clinical reactions to CM reporting a prevalence of adverse reactions from 0–25% [9–13]. Components of hydrolysed CM formulas have been shown to bind IgE

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of children with CMA [14]. In infants, amino acid-derived formulas have been found to successfully manage allergies to extensively hydrolysed protein hydrolysate [15,16]. However, small amounts of intact CM proteins have been found even in such formulas [17]. It has been recommended that a formula should be called 'hypoallergenic' if it is tolerated by at least 90% (with 95% confidence) of the infants with CMA [18]. These criteria have been met by some extensive hydrolysates [9,19] and amino acid mixtures [10].

The aim of the present study was to determine the tolerance and antigenicity of three different protein hydrolysate formulas and of an amino acid-derived formula in a group of children with IgE-mediated CMA.

Methods

Twenty children (12 males and eight females; mean age 2 years 7 months, range 11 months to 9 years) with CMA were enrolled in the study group. CM hypersensitivity was documented by positive food challenges. Urticaria occurred in 12 children, angioedema in eight, atopic dermatitis in seven, rhinitis in two, wheezing in one, and abdominal pain in one. All children had positive skin prick test reactions to whole CM and its components, and milk-specific IgE antibodies (Table 1). The control group consisted of four children (two males, two females; mean age 2 years 1 month, range 13 months to 3 years 4 months) without CMA with negative skin prick tests to CM and negative milk-specific IgE antibodies. Both groups underwent oral challenge tests, skin prick tests and determination of specific IgE antibodies to the following CM substitutes:

extensively hydrolysed whey formula (Hypolac, Alk, Lainate, Milano, Italy) (We), partially hydrolysed whey formula (Humana HA, Humana, Milano, Italy) (Wp), extensively hydrolysed casein formula (Nutramigen, Mead Johnson, Roma, Italy) (Ce), amino acid-derived formula (Nutri-junior, Nutricia, Milano, Italy) (AA). Oral challenge tests to CM substitutes were performed within 5 months from positive challenge tests to CM and were completed in each child in 4 weeks. Informed consent was obtained from parents of all children.

Skin prick test

All children were skin prick tested with undiluted pasteurized CM and with commercial extracts (1:20,w:v) (Bayropharm, Milan, Italy) of whole CM, casein, β -lactoglobulin, α -lactalbumin. In addition, We, Wp, Ce and AA diluted at 2% were tested. The size of the skin test was compared with diluent control and histamine chloride (1 mg/mL). Skin prick tests (SPT) were considered positive if the diameter of the weal was greater than 3 mm, after subtracting the diameter of the weal of the diluent, and was graded in comparison with the histamine weal: + when the weal was half of the histamine weal; ++ when the weal was equal to the histamine weal; +++ when the weal was twice the histamine weal; ++++ when the weal was more than twice the histamine weal [20].

Circulant IgE antibodies

Serum specific IgE antibodies to CM, casein, β -lactoglobulin and α -lactalbumin were determined by CAP (Pharmacia, Uppsala, Sweden). Serum IgE antibodies to We, Wp, Ce and AA were determined by indirect enzyme-linked immunosorbent assay (ELISA). The ELISAs were performed as follows: round-

Table 1. Results of serum total IgE, circulant IgE antibodies and skin prick test (SPT) reactions to whole cow's milk, cow's milk proteins, Wp (partially hydrolysed whey formula), Ce (extensively hydrolysed casein formula), We (extensively hydrolysed whey formula), and AA (amino acid-based formula)

Case	Total IgE (UI/mL)	Cow's milk		α -lactalbumin		β -lactoglobulin		Casein		Wp		Ce		We		AA	
		SPT	IgE (class)	SPT	IgE (class)	SPT	IgE (class)	SPT	IgE	SPT	IgE	SPT	IgE	SPT	IgE	SPT	IgE
1	665	++++	2	-	0	++++	2	-	0	-	-	-	-	-	-	-	-
2	102	++++	3	++++	3	++++	2	++	3	++++	-	-	-	+++	-	-	-
3	29	++++	0	+++	0	+	0	-	0	-	-	-	-	-	-	-	-
4	1958	+++	2	++++	3	+++	0	+++	2	-	-	-	-	-	-	-	-
5	315	++++	2	++++	3	+	2	-	2	-	-	-	-	-	-	-	-
6	508	++++	4	++++	4	++++	3	+++	3	++	-	-	-	-	-	-	-
7	80	++++	2	++++	3	+	2	+	1	+++	+	+	+	++++	+	-	-
8	85	++++	3	++++	3	++++	3	++++	3	++	-	-	-	-	-	+	-
9	460	++++	1	++++	3	++	2	++	1	++	-	-	-	-	-	-	-
10	7	+++	3	-	2	-	1	++	2	++	-	-	-	-	+	-	-
11	50	++++	1	-	0	++++	0	+++	1	+	-	-	-	-	-	-	-
12	205	+++	3	+	0	++	0	+++	0	+	-	-	-	-	-	-	-
13	288	+	5	-	1	-	1	+	4	-	-	-	-	-	-	-	-
14	83	+	0	++	0	-	0	-	0	-	-	-	-	-	-	-	-
15	124	+	1	+++	0	+	0	-	0	+	-	-	-	-	-	-	-
16	166	++++	3	+	0	++++	0	+	0	-	-	-	-	-	-	-	-
17	910	++	6	-	5	-	2	++	6	+++	-	+	-	+	-	-	-
18	152	+++	3	++++	2	++	2	++	3	-	-	-	-	-	-	-	-
19	162	++	2	++	0	++	0	+++	3	++	-	-	-	-	-	-	-
20	113	+++	0	-	0	++++	0	+	0	-	-	-	-	-	-	-	-

bottom polystyrene microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with CM, individual milk proteins (Sigma, St Louis, MO, USA) and hypoallergenic formulas by incubating the wells overnight with 200 mL of a solution containing 5 mgr/mL of the corresponding fraction protein in 0.05 M carbonate-bicarbonate buffer. After three washings with 200 mL of phosphate buffer saline (PBS) containing 0.05% Tween 20, 200 mL of an appropriate dilution of serum samples from normal controls and from atopic subjects in PBS containing 5% horse serum samples were added to the wells. After a 2-h incubation at room temperature (22°C) followed by three washings, 200 mL of an appropriate dilution of peroxidase-conjugated rabbit anti-human IgE (Dako, Copenhagen, Denmark) in PBS containing 5% horse serum were added to the wells. After another 2-h incubation at room temperature followed by three washings, 200 mL of enzyme substrate solution (2 mg/mL ophenylendiamine and 0.015% H₂O₂ in 0.1 M phosphate buffer pH 6.0) were added, absorbance at 492 nm recorded and the results expressed as optical density values. The assay was performed in duplicate and measurements were averaged. Results were considered positive when the absorbance was greater than mean + 2SD of the normal controls. Total serum IgE antibodies were measured by PRIST (Pharmacia).

Food challenge tests

Open food challenge tests were performed in infants, and double-blind, placebo-controlled food challenges in children over 18 months of age [21]. All challenges were performed in a randomised order at the hospital. No children had atopic symptoms, particularly atopic dermatitis, diarrhoea or vomiting in the 3 months before food challenge tests. In this period, all children took soya formula. Therefore, a soya formula, to which children were tolerant, was used as placebo. Children were administered increasing doses of foods or placebo every 20 min on different days. The total amount of food was equal to the average daily intake. Patients were observed for 5 h. If no symptom occurred, parents gave the children the food at home for 4 days. Parents filled out a diary card of symptoms. At the onset of a reaction parents notified the physicians. At the end of

the period each child was visited. Atopic eczema was defined according to the criteria of Hanifin and Rajka [22]. Diarrhoea was defined as the occurrence of three or more liquid or semi-liquid motions which was not overtly caused by an infection (i.e. accompanied by fever or diagnosed by a doctor as gastro-enteritis) [23]. Vomiting was defined as the forceful expulsion of gastric contents, which was not overtly caused by an infection (that is accompanied by fever or diagnosed by a doctor as gastro-enteritis). Children with a convincing history of a severe immediate allergic reaction to CM and a positive skin prick test result were not challenged with fresh milk.

Statistics

The McNemar's test with Yates's correction was used to compare variables with dichotomous data. Results were considered statistically significant at $P < 0.05$. To determine the expected safety of Wp, We, Ce and AA on the basis of the sample size, we calculated binomial confidence intervals.

Results

Food challenge tests

Fifty-nine food challenges were performed in the 20 children included in the study. Sixteen (80%) children were challenged to Ce, 17 (85%) children to Wp, 16 (80%) to We, and 10 (50%) to AA. The challenge tests to CM substitutes were not performed in all children because of lack of compliance. Symptoms developed after 11 (18%) out of 59 food challenges in nine children (Table 2). Five (29%) of 17 children reacted to Wp, three (18%) of 16 to We, two (20%) of 10 to AA, one (6%) of 16 to Ce. Using confidence intervals generated for a binomial (reaction/no reaction) outcome, this study provides 95% confidence intervals that Ce will be tolerated by at least 90% of children with CMA. Neither Wp, nor We, nor AA had an effectiveness of at least 90%. All four substitutes of CM provoked significantly fewer clinical reactions than CM (Wp, $P < 0.001$; We, $P < 0.001$; Ce, $P < 0.001$; AA, $P < 0.005$).

The number of children with clinical reactions to We, Wp, Ce or AA was similar; the differences were statistically not

Table 2. Early (< 12 h) and delayed (> 12 h) symptoms provoked by positive challenge tests to Wp (partially hydrolysed whey formula), Ce (extensively hydrolysed casein formula), We (extensively hydrolysed whey formula), AA (amino acid-based formula) and results of skin prick tests (SPT) and specific IgE antibodies

Case	Formula	Symptoms		SPT	IgE
		Early	Delayed		
5	We	Vomiting	Diarrhoea	–	NEG
7	Ce	Diarrhoea	–	+	POS
8	Wp	Urticaria-angioedema, dyspnoea, hypotension	Eczema	++	NEG
9	Wp	Urticaria-angioedema, dyspnoea, hypotension	Eczema	++	NEG
15	We	Urticaria	–	–	NEG
16	Wp	–	Eczema	–	NEG
17	AA	–	Eczema	–	NEG
19	Wp	Urticaria	–	++	NEG
20	We	–	Eczema	–	NEG
20	Wp	–	Eczema	–	NEG
20	AA	–	Eczema	–	NEG

NEG, negative; POS, positive.

significant. Table 2 lists symptoms that occurred during food challenges. Food challenges elicited isolated early symptoms in three cases, early and delayed symptoms in three cases, isolated delayed symptoms in five cases.

All the placebo challenges gave negative results.

Children who had delayed reactions were followed up to have confirmation of the positive formula challenge. Neither eczematous lesions, nor diarrhoea appeared on an offending formula-free diet during a 6-month follow-up.

Immunologic studies

All the 20 children had positive SPT reactions to CM, 15 (75%) to casein, 16 (80%) to β -lactoglobulin, 14 (70%) to α -lactalbumin (Table 1).

SPT results to We were positive in three (15%) children, to Wp in 11 (55%), to Ce in two (10%), to AA in one (5%) (Table 1). All four substitutes of CM provoked significantly fewer positive SPT reactions than CM (Wp, $P < 0.01$; We, $P < 0.001$; Ce, $P < 0.001$; AA, $P < 0.001$). Wp elicited a significantly higher number of positive SPT reactions than We ($P < 0.025$), Ce ($P < 0.025$) and AA ($P < 0.005$). The number of children with positive SPT reactions to We, Ce and AA was similar; the differences were statistically not significant. In the control group SPT reactions were negative.

Eighteen (90%) out of 20 children had serum CM-specific IgE antibodies, 13 (65%) to casein, 12 (60%) to β -lactoglobulin, 11 (55%) to α -lactalbumin (Table 1).

There was no difference between SPTs and serum IgE antibodies in the number of children with positive results to CM or CM proteins.

Two children had positive specific IgE antibodies to We, one to Wp, one to Ce and none to AA (Table 1). IgE antibodies to CM were found more frequently than those to Wp ($P < 0.001$), We ($P < 0.001$) or Ce ($P < 0.001$). There was no significant difference in the number of children with IgE antibodies to Wp, We, Ce or AA.

There was no difference between SPTs and specific IgE antibodies in the number of children with positive results to CM substitutes. All children with isolated delayed clinical reactions had negative SPTs and specific IgE antibodies to the offending formulas (Table 2).

In the control group, IgE antibodies to CM, CM proteins and CM substitutes were negative.

Discussion

In this study, different hydrolysed CM protein formulas provoked early and delayed clinical reactions, amino acid-based formula only delayed reactions, in children with CMA. There was no significant association between the number of clinical reactions and administration of different CM hydrolysates or amino acid-derived formula. However, only extensively hydrolysed casein formula should be regarded as 'hypoallergic' since it was tolerated with 95% confidence by at least 90% of children allergic to CM [18].

Amino acid-based formula

To our knowledge, this is the first study which reports delayed adverse reactions to an amino acid-derived formula. Our results are at variance from previous studies which found that children

with IgE-mediated CMA tolerated amino acid-based mixtures [10,15]. However, it has been hypothesized that a girl allergic to CM hydrolysates developed urticaria after ingestion of an amino acid-based formula [24]. Amino acid-based formulas are proposed for children who are intolerant to products based on extensively hydrolysed proteins [1,16]. Conversely, we found a child who reacted both to extensively hydrolysed protein formula and amino acid-based formula. A possible explanation for our observations is that we used a commercial formula (Nutri-junior) which was different from that (Neocate, Nutricia) tested in previous studies [10,15,16] which was tolerated by 90% of the children with IgE-mediated CMA (95% confidence) [10]. Mean β -lactoglobulin concentrations in Nutri-junior is higher than in both Neocate and extensively hydrolysed formulas [17]. It is therefore not surprising that we found several clinical reactions to Nutri-junior. According to the manufacturer, the same equipment is used for producing both Nutri-junior and hydrolysed formulas. Therefore, the most likely explanation for the greater amount of mean β -lactoglobulin in Nutri-junior is contamination of the formula during manufacturing.

Hydrolysed protein formulas

Since case reports of anaphylactic reactions after administration of partially whey hydrolysed formulas [5], extensively whey hydrolysed formulas [6] and extensively casein hydrolysed formulas [7,8] in children with CMA have been published, many investigators have evaluated the safety of hydrolysed CM protein formulas. Varying proportions of children who reacted to these formulas have been reported. Differences in previous surveys may depend upon variations in the study design, immunologic mechanism of CMA, patient selection and diagnostic criteria.

Some studies have assessed the tolerance of hydrolysed CM protein formulas in children with non-IgE-mediated CMA by open food challenges that were performed at home [25–27]. Taking together the results of these studies, extensively hydrolysed casein formulas were reported to induce clinical hypersensitivity reactions in two (9%) of 22 children, extensively hydrolysed whey formulas in four (3%) of 105. Vanderhoof et al. [28] studied 28 infants with formula-protein induced colitis without IgE-mediated CM hypersensitivity. Twenty of them had symptoms after extensively hydrolysed casein formulas administration and 3/28 had symptoms after amino acid-derived formula administration.

Two studies investigated the safety of CM substitutes in children with IgE-mediated CMA by open challenges. Martin-Esteban [19] found that 2/33 children with IgE-mediated CMA reacted to a mixture of casein and whey protein hydrolysate (Damira, Novartis). de Boissieu et al. [16] selected 13 children with highly IgE-mediated CMA. Six were intolerant to extensively hydrolysed casein formulas and seven to extensively hydrolysed whey formulas. All 13 children tolerated an amino acid-based formula.

Some studies have assessed the safety of CM protein hydrolysate formulas on the basis of blind or double-blind food challenges in children with IgE-mediated CMA [9–13,15]. In most reports, extensively hydrolysed formulas were used as placebo to study the tolerance either to CM or to CM substitutes.

Combining the results of these studies [9–13,15], extensively hydrolysed casein formulas induced clinical hypersensitivity

reactions in five (6%) of 80 children, extensively hydrolysed whey formulas induced reactions in four (17%) of 23, and reactions were induced by partially hydrolysed whey formula in 14 (50%) of 28. We obtained prevalences of positive challenge tests to extensively hydrolysed casein, extensively hydrolysed whey and partially hydrolysed whey formulas in children with CMA very similar to those reported in previous studies. Our results confirm that only extensively hydrolysed casein formula should be considered 'hypoallergic', since it is tolerated by at least 90% of children (95% confidence) [9]. As underlined above, children with intolerance to CM seem to show a more favourable response to extensively hydrolysed whey formulas.

Formulas with moderately reduced allergenicity are marketed for the prevention of allergy in some countries. In our study, we could demonstrate that Wp has a considerable residual allergenic activity and it is not safe for the treatment of CMA since a high number of children react to this formula. This supports the need of more studies on the preventive effects of such formula, before recommending its administration in infants.

In this study, we did not succeed in performing challenge tests to all formulas in all children because of lack of compliance. We think that the main reason for this is the necessity of performing many challenge tests over a short period of time because hypersensitivity to CM is progressively lost in children. However, since the order of the challenge tests was randomised we believe that this limitation did not introduce any significant bias in this study.

Commercially available products based on partially hydrolysed proteins or extensively hydrolysed proteins contain residual amounts of antigenically active molecules derived from CM [14,17,29]. Furthermore, new allergenic epitopes may result from the enzymatic hydrolysis and heat treatment during manufacturing [30]. The mechanisms by which epitopes of these residual peptides or new epitopes provoke the onset of adverse reactions is not fully elucidated. Our findings show by *in vivo* and *in vitro* methods that there is a IgE-mediated reaction to CM substitutes. Our data corroborate previous findings showing IgE-binding to components of hydrolysed CM formulas in sera from children with CMA [14]. Therefore, we think that some clinical reactions may be the result of immediate IgE-mediated reactions. We observed in children with delayed reactions that SPTs and specific IgE antibodies to the offending formulas are negative. One explanation may be the lack of sensitivity of the tests. Another explanation may be that adverse reactions were not IgE-mediated or non-immunologically mediated.

Conclusion

Our findings show that most children with CMA are able to tolerate extensively hydrolysed protein formulas, especially based on casein, and amino acid-derived formula. Since we identified highly sensitized children who had immediate clinical hypersensitivity reactions to extensively hydrolysed protein formulas, we suggest that children with CMA should be treated with such formulas under medical supervision to document tolerance. Our data do not indicate that first ingestions of amino acid mixtures require close medical surveillance.

However, all children with CMA should be monitored after intake of CM substitutes for several days because of delayed reactions.

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