

Immunogenicity and antigenicity of a partially hydrolyzed cow's milk infant formula

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We evaluated the immunogenicity and antigenicity of a formula based on partially hydrolyzed cow's milk whey protein in infants at risk of atopy and in controls. Total IgE and specific IgE, IgG, and IgG4 subclass antibodies against egg albumin and cow's milk α -lactalbumin, casein, and β -lactoglobulin were measured by radioimmunoassay of cord blood and of peripheral blood at 5 days and 6 months of life in five groups of infants: 16 breast-fed infants at risk of atopy (group 1), 21 partially hydrolyzed whey formula-fed infants at risk of atopy (group 2), 14 formula-fed infants at risk of atopy (group 3), 10 breast-fed control infants (group 4), and 13 formula-fed control infants (group 5). Total IgE concentration was significantly lower in group 2 at 6 months than in groups 3 and 5 infants and similar to that observed in groups 1 and 4 infants. The concentration of specific anti-egg and anti-cow's milk protein IgG and of specific anti-cow's milk α -lactalbumin and β -lactoglobulin IgG4 subclass antibodies was significantly reduced in group 2 as compared to group 3 infants and similar to that found in breast-fed infants. In conclusion, the partially hydrolyzed formula was less immunogenic and antigenic than a traditional formula and was as immunogenic and antigenic as breast milk.

Key words: antigenicity; atopy; casein; egg albumin; hydrolyzed milk formula; IgE; IgG; immunogenicity; α -lactalbumin; β -lactoglobulin.

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An increased incidence of allergic disorders has been reported over the last two decades (1): more than 20% of children are estimated to have allergic manifestations (eczema, asthma, food intolerance, rhinitis) at 2 years of age (2), and the cumulative incidence of atopic symptoms is about 35–40% by the age of 5 years (3).

Several authors have suggested prevention strategies based on the early recognition of subjects at risk and on allergen avoidance, particularly during the first 6–12 months of life. The early recognition of infants at risk of atopic disease is mainly based on a positive family history of atopy and, to a lesser extent, due to the low sensitivity, on the measurement of cord-blood IgE concentration. Allergen-avoidance programs include: prolonged exclusive breast-feeding, low-antigen diet in the lactating woman (4–7), delayed weaning, avoidance of passive smoking, reduction of exposure to pets and mites, and avoidance of nurseries.

These programs have usually been successful in reducing or, at least, in retarding the onset of allergic symptoms.

When the mother cannot breast-feed her infant, the use of a special formula is usually suggested (7–11); however, there is disagreement on which kind of special formula is best for the prevention of atopic disorders. Soy-protein formulas have often been found to cause allergy (12), while formulas based on extensively hydrolyzed cow's milk protein have frequently been unsatisfactory because of their high cost and low palatability, leading to problems of compliance.

Preliminary studies have reported that the less expensive and more palatable formulas based on partially hydrolyzed cow's milk protein could be effective for the prevention of atopic disorders in high-risk infants (13–17); however, few data are available on the effects of these formulas on the immune response.

The present study aimed to evaluate the immunogenicity and antigenicity of a formula based on partially hydrolyzed cow's milk whey protein in infants at risk of atopy and in controls.

Material and methods

We evaluated five groups of healthy term infants consecutively born in our division from September 1993 to April 1994:

- 1) breast-fed infants at risk of atopy
- 2) partially hydrolyzed whey formula-fed infants at risk of atopy
- 3) formula-fed infants at risk of atopy
- 4) breast-fed control infants
- 5) formula-fed control infants.

An infant was considered at risk of atopy if at least the mother had a positive clinical history of atopic disease (rhinitis, asthma, eczema, food intolerance) confirmed by skin tests, challenge test, or laboratory investigations.

Breast-feeding was strongly encouraged for all infants. After informed consent was obtained from the parents, the infants who were considered at risk of atopy, and who could not be breast-fed, were randomized (by the sealed-envelope method) in the first day of life to receive either a traditional milk formula or the protein hydrolysate formula.

The following allergen-avoidance program was suggested for the infants at risk: dietary restriction (avoidance of cow's milk and derivatives, fish, eggs, and peanuts and derivatives) in the lactating mother, delayed weaning (at 6 months of age), avoidance of passive smoking, reduction of exposure to pets and mites, and avoidance of nurseries. In the control infants, the only recommendations were to delay weaning until after the sixth month and to avoid passive smoking.

All the infants were closely followed up every 2 weeks for at least 6 months to evaluate compliance with the feeding regimen, growth and development, and onset of symptoms of atopy. The amount of formula consumed by infants in groups 2, 3, and 5 was recorded. Only the infants who were exclusively breast-fed, special formula-fed, or traditional formula-fed and who were not exposed to passive smoking during the first 6 months of life were included in the study.

The proteins present in the partially hydrolyzed whey formula (Vivina HA-Primigiorni HA, Plada, Milan, Italy) used in the present study, as tested by polyacrylamide gel electrophoresis, were reported to be composed of 36% peptide fractions with molecular mass <1 kDa, 30% 1–2 kDa, 29% 2–6 kDa, and 5% >6 kDa.

During routine hematologic investigations, samples of 1 ml of cord blood and of peripheral blood at 5 days and 6 months of age were obtained from all infants for measurement of total IgE and specific IgE, IgG, and IgG4 subclass antibodies against egg albumin and cow's milk α -lactalbumin, casein, and β -lactoglobulin. Blood samples were centrifuged and sera collected and stored at -20°C until analysis.

Total IgE, specific IgE, and specific IgG and IgG4 subclass antibodies were measured by the Pharmacia CAP System RAST radioimmunoassay (Pharmacia, Uppsala, Sweden).

Briefly, the principle of the procedure was the following. The allergen of interest (or anti-IgE antibodies for total IgE measurement), covalently coupled to ImmunoCAP, reacts with the specific IgE, IgG, or IgG4 (or total IgE) in the patient serum specimen. After washing away nonspecific IgE, IgG, and IgG4, ^{125}I -labeled antibodies against IgE, IgG, or IgG4 were added to form a complex. After incubation, unbound, radioactively labeled anti-IgE, anti-IgG, or anti-IgG4 was washed away, and the radioactivity of the bound complex was measured in a gamma counter. The more radioactivity bound, the more total IgE or specific IgE, IgG, or IgG4 is present in the specimen. For classification of test results, counts for patient samples were compared directly with counts for standards run in parallel; IgE standards were calibrated against the Second International Reference Preparation 75/502 of human serum IgE from the World Health Organization, while IgG and IgG4 were calibrated against a standardized human serum pool with a high content of IgG or IgG4 antibodies for the reference allergen.

Total and specific IgE values were expressed in kU/l; the specific IgE RAST was considered positive with values equal to or greater than 0.35 kU/l. Specific IgG and IgG4 values were expressed in percentage of reference response by the following formula:

$$\frac{\text{mean counts per minute unknown}}{\times 100/\text{mean counts per minute reference}}$$

The person who measured the antibody concentration was unaware of the type of feeding or of the age of the infant from whom the sample had been obtained.

The study was approved by the ethics committee of the Hospital of Pavia.

Statistics

The statistical analysis used the two-way analysis of variance (ANOVA) for repeated measures, followed

by the "post hoc" Newman-Keuls test, to evaluate differences in antibody concentration among the five groups of infants at different ages. A *P* value >0.05 was considered not significant. All computations were performed with a commercial statistical package (STATISTICA for Windows, StatSoft, Inc, Tulsa, OK, USA).

Results

At the end of the study, the numbers of infants who met the criteria (the same type of feeding, no weaning until 6 months, and no exposure to passive smoking) for inclusion in the five groups were 16 in group 1 (breast-fed infants at risk of atopy), 21 in group 2 (special formula-fed infants at risk of atopy), 14 in group 3 (formula-fed infants at risk of atopy), 10 in group 4 (breast-fed control infants), and 13 in group 5 (formula-fed control infants).

None of the infants had significant feeding problems or infections; all grew well and developed normally.

At 6 months, no significant difference was observed in the amounts of milk consumed by the formula-fed infants in groups 2, 3, and 5.

Eczema (defined as a pruritic, chronic, or chronically relapsing dermatitis with typical features and distribution) (18) was observed in four infants of group 1 and two of group 3.

RAST was positive for milk or egg proteins in five infants of group 1 (positive for casein in one patient, β -lactoglobulin in one patient, egg albumin in two patients, and egg albumin, casein, and α -lactalbumin in one patient), in one infant of group 2 (positive for egg albumin and α -lactalbumin), in one infant of group 3 (positive for egg albumin), in two infants of group 4 (positive for egg albumin and casein in one patient and casein in another one), and in three infants of group 5 (positive for α -lactalbumin, egg albumin, and β -lactoglobulin) at 6 months of age; only one infant of group 1 was positive for both RAST and eczema.

The results for total IgE and specific IgG and IgG4 subclass antibodies serum levels against egg albumin and cow's milk proteins and the results of the statistical analysis are reported in Tables 1–9.

No significant differences were observed between serum levels of total IgE and specific immunoglobulins at birth and 5 days. All the specific immunoglobulins except IgG4 against egg albumin and cow's milk casein were significantly increased at 6 months only in group 3. Total IgE concentration was higher at 6 months in formula-fed infants, both those at risk of atopy (group 3) and controls (group 5).

Table 1. Results for specific IgG antibodies serum levels against egg albumin and statistical analysis. Specific IgG values are expressed in percentage of reference response

	Anti-egg-albumin IgG					
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	24.7	16.6	25.2	19.8	13.6	9.5
Group 2	28.4	20.5	32.9	27.7	22.1	23.6
Group 3	25.6	28.0	26.4	23.8	52.7	55.4
Group 4	20.2	11.5	19.8	11.2	18.9	19.9
Group 5	20.6	8.3	19.4	9.8	14.2	8.3

Analysis of variance						
df effect	MS effect	df error	MS error	F	P value	
4	2.014	69	881	2.2872	0.068672	
2	13	138	374	0.0339	0.9667	
8	1.189	138	374	3.1835	0.00241	

Newman-Keuls test: anti-egg-albumin IgG concentration in group 3 at 6 months was significantly higher ($P=0.01$) than that of same group in cord blood and at 5 days and that of other four groups at 6 months (df = degree of freedom; MS = mean square).

Table 2. Results for specific IgG antibodies serum levels against cow's milk α -lactalbumin and statistical analysis. Specific IgG values are expressed in percentage of reference response

	Anti- α -lactalbumin IgG					
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	24.0	9.1	23.5	7.8	16.2	7.7
Group 2	20.7	11.8	19.2	9.4	30.1	22.3
Group 3	25.1	15.4	22.0	9.0	83.6	51.5
Group 4	25.5	12.9	20.7	10.7	39.5	33.5
Group 5	26.0	11.5	21.7	12.0	29.7	20.8

Analysis of variance						
df effect	MS effect	df error	MS error	F	P value	
4	3476.405	69	506.4397	6.8644	0.000104	
2	6826.627	138	306.7587	22.2541	0.0000000	
8	3185.971	138	306.7587	10.3959	0.0000000	

Newman-Keuls test: anti- α -lactalbumin IgG concentration in group 3 at 6 months was significantly higher ($P=0.01$) than that of same group in cord blood and at 5 days and that of other four groups at 6 months.

Discussion

Piacentini et al. (19) recently reported that the same partially hydrolyzed milk formula used in the present study caused less sensitization than a traditional formula in a guinea-pig model, as demonstrated by a reduced incidence of severe reactions to milk proteins after challenge and by lower

Table 3. Results for specific IgG antibodies serum levels against cow's milk β -lactoglobulin and statistical analysis. Specific IgG values are expressed in percentage of reference response

Anti- β -lactoglobulin IgG						
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	25.7	10.2	25.5	10.1	17.3	10.8
Group 2	28.3	16.0	25.9	13.4	29.5	21.9
Group 3	22.2	8.2	20.7	7.1	68.8	45.7
Group 4	26.0	15.5	23.3	14.5	26.1	19.4
Group 5	25.5	10.7	21.2	9.0	33.2	42.9

Analysis of variance						
df effect	MS effect	df error	MS error	F	P value	
4	1269.678	69	522.866	2.4668	0.052839	
2	2631.596	138	344.7218	7.6340	0.000717	
8	2253.696	138	344.7218	6.5377	0.000000	

Newman-Keuls test: anti- β -lactoglobulin IgG concentration in group 3 at 6 months was significantly higher ($P=0.01$) than that of same group in cord blood and at 5 days and that of other four groups at 6 months.

Table 4. Results for specific IgG antibodies serum levels against cow's milk casein and statistical analysis. Specific IgG values are expressed in percentage of reference response

Anti-casein IgG						
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	30.6	25.2	31.7	26.7	34.2	28.0
Group 2	36.4	31.8	33.8	25.6	43.6	25.4
Group 3	33.9	24.1	28.4	22.8	93.4	41.4
Group 4	25.3	13.5	22.1	12.3	35.8	21.6
Group 5	26.5	11.7	21.1	9.0	49.6	37.5

Analysis of variance						
df effect	MS effect	df error	MS error	F	P value	
4	3472.479	69	1029.8	3.3720	0.01403	
2	11787.98	138	509.661	23.1291	0.000000	
8	2811.382	138	509.661	5.5162	0.000005	

Newman-Keuls test: anticasein IgG concentration in group 3 at 6 months was significantly higher ($P=0.01$) than that of same group in cord blood and at 5 days and that of other four groups at 6 months.

production of anti-milk-protein IgG in animals fed the partially hydrolyzed milk formula.

In the present study, we evaluated five groups of infants with or without a positive family history of atopic disease who were exclusively fed only one kind of milk (maternal, partially hydrolyzed milk formula, or traditional milk formula) during the first 6 months of life. We observed that the partially

Table 5. Results for specific IgG4 antibodies serum levels against egg albumin and statistical analysis. Specific IgG4 values are expressed in percentage of reference response

Anti-egg-albumin IgG4						
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	9.4	19.6	10.2	23.2	3.4	0.8
Group 2	13.4	18.1	12.3	16.7	7.2	14.1
Group 3	17.0	26.6	13.7	21.6	15.0	28.6
Group 4	5.0	2.6	4.5	1.3	3.0	0.7
Group 5	6.9	5.2	5.6	4.0	3.8	1.3

Analysis of variance						
df effect	MS effect	df error	MS error	F	P value	
4	791.4764	69	451.9221	1.7514	0.149072	
2	281.6337	138	183.2919	1.5365	0.218784	
8	42.19414	138	183.2919	0.2302	0.98477	

No significant differences of anti-egg-albumin IgG4 concentration were observed between or within groups.

Table 6. Results for specific IgG4 antibodies serum levels against cow's milk α -lactalbumin and statistical analysis. Specific IgG4 values are expressed in percentage of reference response

Anti- α -lactalbumin IgG4						
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	8.9	12.4	9.4	13.3	3.7	1.1
Group 2	4.1	3.0	3.8	1.8	3.5	1.5
Group 3	9.4	12.2	8.0	7.4	30.7	39.7
Group 4	5.3	5.0	3.6	2.1	5.2	2.0
Group 5	7.8	9.9	5.3	6.8	6.0	4.5

Analysis of variance						
df effect	MS effect	df error	MS error	F	P value	
4	1050.25	69	187.648	5.5869	0.000582	
2	267.8709	138	128.7565	2.0804	0.128774	
8	558.5859	138	128.7565	4.3383	0.000109	

Newman-Keuls test: anti- α -lactalbumin IgG4 concentration in group 3 at 6 months was significantly higher ($P=0.01$) than that of same group in cord blood and at 5 days and that of other four groups at 6 months.

hydrolyzed milk formula was less immunogenic than the traditional milk formula and as immunogenic as breast milk; in fact, total IgE concentration was significantly lower in group 2 at 6 months than in groups 3 and 5 and similar to that observed in groups 1 and 4.

Furthermore, we observed reduced antigenicity of the partially hydrolyzed milk formula; in fact, the production of specific IgG anti-egg and anti-cow's

Table 7. Results for specific IgG4 antibodies serum levels against cow's milk β -lactoglobulin and statistical analysis. Specific IgG4 values are expressed in percentage of reference response

	Anti- β -lactoglobulin IgG4					
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	6.4	7.2	8.4	11.0	3.6	1.4
Group 2	7.8	7.8	7.1	6.2	4.5	1.8
Group 3	5.4	3.4	5.2	3.8	22.0	25.0
Group 4	4.9	3.3	3.8	1.9	4.0	1.2
Group 5	9.0	7.2	5.4	3.9	12.3	27.9

Analysis of variance					
df effect	MS effect	df error	MS error	F	P value
4	254.955	69	120.3053	2.1192	0.087658
2	212.9514	138	109.6064	1.9429	0.147191
8	367.5342	138	109.6064	3.3532	0.001533

Newman-Keuls test: anti- β -lactoglobulin IgG4 concentration in group 3 at 6 months was significantly higher ($P=0.01$) than that of same group in cord blood and at 5 days and that of other four groups at 6 months.

Table 8. Results for specific IgG4 antibodies serum levels against cow's milk casein and statistical analysis. Specific IgG4 values are expressed in percentage of reference response

	Anti-casein IgG4					
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	9.1	21.0	11.7	24.4	3.5	1.4
Group 2	13.6	26.0	11.1	19.6	4.2	1.6
Group 3	14.9	17.4	10.4	10.7	18.6	15.9
Group 4	3.8	2.2	3.2	1.7	4.1	1.3
Group 5	7.0	7.9	5.3	5.9	5.7	3.7

Analysis of variance					
df effect	MS effect	df error	MS error	F	P value
4	648.2933	69	402.9278	1.6090	0.181858
2	107.4796	138	138.702	0.7749	0.462745
8	202.726	138	138.702	1.4616	0.176738

No significant differences in anticasein IgG4 concentration were observed between or within groups.

milk proteins and of specific anti-cow's milk α -lactalbumin and β -lactoglobulin IgG4 subclass antibodies was reduced in group 2 infants at 6 months, as compared to group 3 infants, and was similar to that found in breast-fed infants. The traditional cow's milk formula was more immunogenic both in infants at risk of atopy and in controls, and more antigenic only in infants at risk of atopy.

Table 9. Results for total IgE serum levels and statistical analysis. Values are expressed in kU/l

	Total IgE					
	Cord blood		5 days		6 months	
	Mean	SD	Mean	SD	Mean	SD
Group 1	0.4	0.0	0.4	0.0	6.2	6.0
Group 2	0.4	0.2	0.4	0.1	7.6	8.9
Group 3	0.5	0.5	0.4	0.0	28.0	40.4
Group 4	0.5	0.3	0.4	0.0	7.6	7.0
Group 5	0.5	0.4	0.7	0.6	20.8	33.7

Analysis of variance					
df effect	MS effect	df error	MS error	F	P value
4	471.4041	69	182.2273	2.5869	0.043227
2	4314.005	138	180.2072	23.939	0.000000
8	456.3349	138	180.2072	2.5323	0.013305

Newman-Keuls test: total IgE concentration in groups 3 and 5 at 6 months was significantly higher ($P=0.01$) than that of same group in cord blood and at 5 days and that of other three groups at 6 months.

Reduced immunogenicity can be considered a potentially favorable effect, as several reports have correlated high IgE serum levels with the appearance of symptoms of atopy (18); on the other hand, it is not clear whether the reduced antigenicity of the partially hydrolyzed milk formula can be regarded as a positive, negative, or clinically unimportance effect; in fact, both IgG and IgG4 subclass specific antibodies have been involved either in the induction of tolerance of food antigens or in the pathogenesis of allergic reactions (20–24).

As for clinical efficacy, the partially hydrolyzed milk formulas cannot be used in children who are already allergic to cow's milk; however, preliminary studies have reported that these formulas may have a beneficial effect in reducing or delaying the onset of symptoms of atopy. In a double-blind study, Chandra et al. (14) observed a significantly reduced cumulative incidence of atopic eczema and recurrent wheezing until 18 months of age in infants at risk of atopy who were fed a partially hydrolyzed whey formula; these results have been recently confirmed after a 5-year follow-up (13). In a double-blind study, Vandenplas et al. (16) observed that feeding a partially hydrolyzed whey formula during the first 6 months of life to high-risk infants decreased the incidence of atopic disease up to the age of 12 months. Similar results were obtained by Willems et al. (17) and, in an open study, by Marini et al. (15) in infants followed up to 3 years of age.

The controversy on antigen-reduced infant formulas has been animated in the literature, with the

participation of international committees and single researchers (25–30). The above-mentioned preliminary clinical studies have been criticized because of lack of stringent inclusion and diagnostic criteria (25, 30). Moreover, Ragno et al. (31) reported a 45% incidence of allergic reactions in infants with severe cow's milk allergy challenged with a partially hydrolyzed whey formula. Therefore, some authors have discouraged the use of the partially hydrolyzed formulas in favor of the extensively hydrolyzed formulas and have suggested that the term "hypoallergenic" should not be used for these special formulas, in accordance with the American Academy of Pediatrics guidelines (26, 27). On the other hand, other authors believe that, for the prevention of atopic manifestations, the partially hydrolyzed formulas should be preferred to the extensively hydrolyzed formulas because the former are more palatable, cost less, and have more durable effects (4).

In conclusion, our study showed that an infant formula obtained by partially hydrolyzed cow's milk whey was less immunogenic and antigenic than a traditional cow's milk formula, and no more immunogenic and antigenic than breast milk. Large, clinical, double-blind, placebo-controlled studies are necessary to verify the preventive effect on the onset of symptoms of allergy of partially hydrolyzed formulas in infants at very high risk of atopy who cannot be breast-fed, and to evaluate the cost/effectiveness ratio of this preventive measure.

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