

G. Oldæus
B. Björkstén
M.C. Jenmalm
N.-I.M. Kjellman

Cow's milk IgE and IgG antibody responses to cow's milk formulas

Authors' affiliations:

G. Oldæus, Department of Paediatrics,
Länssjukhuset Ryhov, Jönköping
G. Oldæus, B. Björkstén, M.C. Jenmalm, N.-I.M.
Kjellman, Department of Health and
Environment, Division of Paediatrics, University
Hospital, Linköping, Sweden

Correspondence to:

Göran Oldæus
Barn och ungdomsmedicinska kliniken
Länssjukhuset Ryhov
S-551 85 Jönköping
Sweden

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For comparison of the antigenicity and allergenicity of three cow's milk formulas, serum IgE antibodies to cow's milk, β -lactoglobulin and casein, and IgG antibodies to β -lactoglobulin were analyzed in 94 infants with a family history of allergy. They were participating in a randomized trial comparing the allergy prophylactic effect of feeding an extensively hydrolyzed (N), a partially hydrolyzed (PH), and a regular cow's milk formula (RM). Only infants who had been formula-fed for 3 months or more were included. IgE antibodies to cow's milk proteins were more common in the RM group (22/34) than in the N (2/31) and PH groups (3/29). There was a strong correlation between sensitization to cow's milk and β -lactoglobulin ($r_s=0.85$, $P<0.001$). The IgG responses to β -lactoglobulin were low in the N group, intermediate in the PH group, and high in the RM group. High responses, as well as detection of IgE antibodies, were associated with development of atopic disease. The low antigenicity and allergenicity of the extensively hydrolyzed formula support its use in allergy prophylaxis. The partial hydrolysate seemed to be less suitable for this purpose.

Treatment of cow's milk allergy includes total avoidance of cow's milk proteins. Usually, a soy or an extensively hydrolyzed cow's milk formula is given as replacement (1). However, soy formulas may cause allergic sensitization in atopic children, and their use in cow's milk-allergic children has been controversial (2). In recent years, extensively hydrolyzed cow's milk-based formulas (eHF) have been recommended for treatment and prevention of cow's milk allergy (1). The nutritional committee of the American Academy of Pediatrics has recommended that hypoallergenic formulas should be tolerated by 90% of children with cow's milk allergy with 95% confidence (3, 4). Although

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Table 1. Reported cumulative symptoms at 9 months and cumulative diagnoses at 18 months of age. Both probable and obvious diagnoses were included. For gastrointestinal allergy at 9 months, only double-blind, placebo-controlled challenges were included

Age	Formula group	n	Diagnosis				
			Wheeze n (%)	AD n (%)	GIA n (%)	AD+GIA n (%)	Any n (%)
9 months	N	31	5 (16)	5 (16)	0	5 (16)	9 (29)
	PH	29	3 (10)	9 (31)	1 (3)	9 (31)	11 (38)
	RM	34	10 (29)	12 (41)	1 (3)	12 (41)	17 (50)
18 months	N	31	5 (16)	9 (29)	2 (6)	9 (29)	11 (35)
	PH	29	3 (10)	10 (35)	3 (10)	12 (41)	14 (48)
	RM	34	10 (29)	15 (44)	5 (15)	17 (50)	21 (62)

N: extensive hydrolysate; PH: partial hydrolysate; RM: regular formula group; AD: atopic dermatitis; GIA: gastrointestinal allergy; any: any probable or obvious diagnosis of AD, GIA, or wheeze.

successfully used for more than 50 years in cow's milk-allergic children, there are major drawbacks to the extensive hydrolysates, such as their unpalatability and high cost. Partially hydrolyzed formulas (pHF) have been marketed to overcome these problems. Some of these formulas have caused severe allergic reactions in cow's milk-allergic children (5, 6). It has also been demonstrated that pHF have considerable remaining allergenicity (7–10). To some extent, this could be explained by more residual β -lactoglobulin in the pHF than the eHF (8, 11). It has even been suggested that the suitability of substitute formulas marketed for patients with allergy to cow's milk

could be graded according to the β -lactoglobulin content (12). The remaining allergenicity in cow's milk hydrolysates is highly dependent on the degree of enzymatic hydrolysis, choice of enzyme system, source of protein, and various manufacturing procedures, such as filtration and heating (13).

Several studies have found that feeding eHF is associated with a reduced incidence of atopic manifestations during infancy in babies with a family history of allergy (14–17), but only in a few has a reduced cumulative incidence of cow's milk protein allergy or cow's milk-specific IgE antibodies been demonstrated (1).

Partial hydrolysates are not considered to be useful for treatment of cow's milk allergy because of their recognized allergenicity (1). There have been claims, however, of a possible allergy-preventive effect of such formulas in babies with a family history of allergy (18), but controlled studies adhering to the recommended quality criteria of clinical trials (1) are still lacking. Therefore, their use in the prevention of allergy is not recommended (1).

On the hypothesis that the remaining intact cow's milk antigens in formulas would initiate an antibody response to the same antigens in atopy prone-infants, we investigated the cow's milk-specific IgG and IgE antibody response after at least a 3-month feeding period. The possible association between the response and clinical atopic disease was evaluated.

Material and methods

Infants

Serum samples were obtained by venipuncture from infants participating in a controlled prospective random-

Table 2. Prevalence of IgE antibodies to cow's milk proteins at 9 months in infants with family history of allergy who were fed either Nutramigen[®] (N), partial hydrolysate (PH), or ordinary milk formula, Enfamil[®] (RM). Number of infants with blood samples available for analysis (n) and number of infants with detectable antibodies (n+) are given. For comparisons between formula groups, Fisher's exact test was used

	Formulas			P
	N n+/n	PH n+/n	RM n+/n	
IgE-CM	2/30	2/29	21/32	N vs PH NS
				N vs RM <0.001
				PH vs RM <0.001
IgE-BLG	0/31	3/29	19/34	N vs PH NS
				N vs RM <0.001
				PH vs RM <0.001
IgE-casein	0/30	1/29	4/33	N vs PH NS
				N vs RM NS
				PH vs RM NS

IgE-CM: IgE antibodies to cow's milk; IgE-BLG: IgE antibodies to β -lactoglobulin; IgE-casein: IgE antibodies to casein.

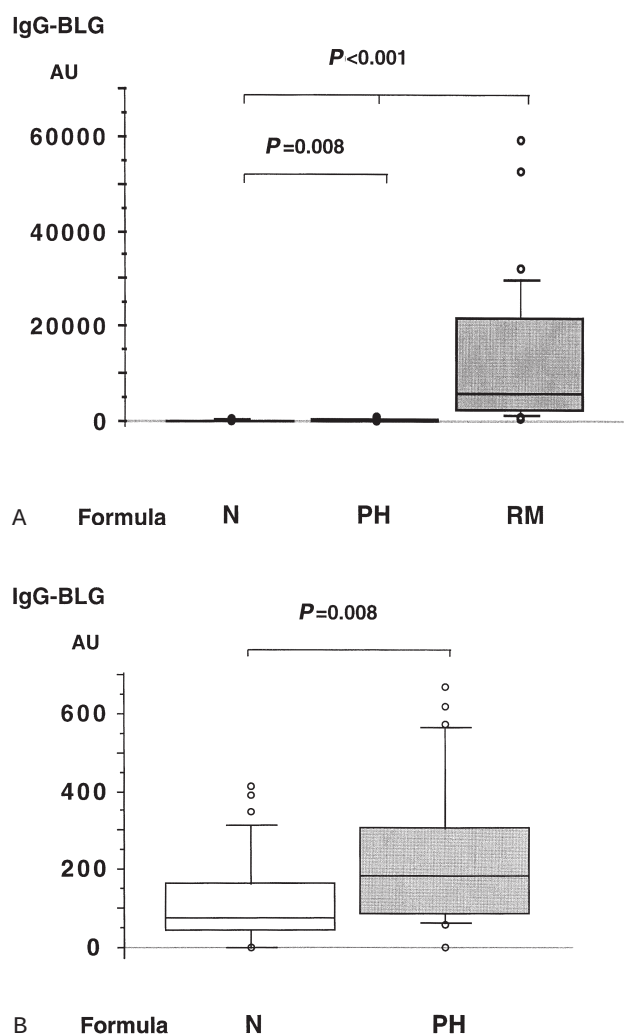


Figure 1A and B. Distribution of IgG antibodies to β -lactoglobulin (BLG) at 9 months of age in infants at risk of subsequent allergy in three feeding groups (A) given either extensive hydrolysate (Nutramigen [N]), partial hydrolysate (PH), or regular formula (RM), and comparison between N and PH groups (B) in enlarged scale. Formulas were given for at least 3 months for prophylactic purposes. 10th, 25th, 50th, 75th, and 90th percentiles are given, as are outliers above 90th percentile and below 10th percentile. Values are given in arbitrary units (AU).

mized allergy prophylaxis study (17). The infants ($n=203$) were all at high risk of developing allergy as defined by a double heredity or a single heredity, combined with cord blood IgE of ≥ 0.5 kU/l (PRIST[®], Pharmacia, Uppsala, Sweden). They were followed from birth to 18 months of age. The allergy-preventive measures included abstention from cow's milk, eggs, and fish by lactating mothers and infants up to 9 months of age. At weaning, infants were randomized to one of three cow's milk-based formulas; an extensively hydrolyzed casein formula (N, Nutramigen[®], Mead Johnson, Evansville, IN, USA), a partially hydrolyzed whey and casein-based formula (PH, Mead Johnson), and a regular formula (RM, Enfamil[®], Mead Johnson). In all, 155

Table 3. Levels of IgE and IgG antibodies to cow's milk proteins at 9 months of age in relation to atopic diagnoses at 9 and 18 months of age in 94 infants independent of feeding group. Both probable and obvious diagnoses were included. Median was <1.0 SU/ml for all IgE determinations. Mean ranks and probability values (Mann-Whitney *U*-test) are given for each group

	Any atopic diagnosis					
	9 months			18 months		
	Yes	No	P	Yes	No	P
IgG-BLG						
<i>n</i>	32	52	39	45		
Median, AU	581	174	407	163		
Mean rank	50.0	37.9	<0.05	49.5	36.4	<0.05
IgE-CM						
<i>n</i>	37	54	45	46		
Mean rank	53.0	41.2	<0.01	52.7	39.5	<0.01
IgE-BLG						
<i>n</i>	38	56	46	48		
Mean rank	53.9	43.2	<0.05	54.1	41.2	<0.01

BLG: β -lactoglobulin; CM: cow's milk.

infants were randomized, and the formula was then given until 9 months of age.

Only infants who had been formula fed for at least 3 months were included in the analysis ($n=113$) to ensure a relevant exposure. Blood samples taken at 9 months of age were available from 94 of them ($n=31$, $n=29$, and $n=34$ in the N, PH, and RM groups, respectively) for analysis of IgE antibodies to cow's milk, β -lactoglobulin and casein, as well as IgG antibodies to β -lactoglobulin. The antibody levels at 9 months of age were related to the clinical outcome at 9 months, as defined by the cumulative incidence of reported atopic symptoms and the double-blind, placebo-controlled formula challenges, and the cumulative diagnosis of probable or obvious atopic disease at 18 months of age (17).

Laboratory analysis

The determinations of IgG antibodies to β -lactoglobulin were done with a sensitive ELISA, as described in detail elsewhere (19). Briefly, Costar 3590 microtiter plates (Life Technologies AB, Täby, Sweden) were coated with β -lactoglobulin (Sigma-Aldrich, Stockholm, Sweden). Free binding sites were blocked with human serum albumin (HSA) (Chemicon, Stockholm, Sweden). Human serum samples or buffer without added serum (blanks) were added

to duplicate wells, and then monoclonal antibodies to human IgG (IgG clone HP 6064) (Bio Zac AB, Järfälla, Sweden) were added. Alkaline-phosphatase-conjugated rabbit antimouse IgG (Sigma-Aldrich) was added, and *p*-nitrophenyl phosphate (pNPP) (Sigma-Aldrich) was used as substrate. The optical density (OD) was read at 405 nm. Values were expressed as arbitrary units (AU) deduced from the ODs of the reference serum curve, after subtracting the blanks. A coefficient of variation (CV) below 15% was accepted for duplicate samples. Intra-assay CVs were of <5%. Interassay CVs were of <15%.

Circulating IgE antibodies to whole cow's milk, bovine β -lactoglobulin, and bovine casein were analyzed with the high sensitivity protocol of the Magic Lite[®] System (ALK Laboratories, Hørsholm, Denmark), an immunochemiluminometric assay (20). With a prolonged incubation overnight, the detection limit was extended from 1.44 to 1.0 SU/ml. A variation between duplicate samples of less than 20% was accepted; otherwise, a new analysis was performed. According to the manufacturer (ALK), 1.44–4.0 SU/ml corresponds to 0.36–0.7 kU/l in the CAP RAST[®] System (Pharmacia, Uppsala, Sweden), and 100 SU/ml to 17.5 kU/l.

All analyses were done after completion of the feeding periods with no knowledge of any clinical data.

Statistics

As the results were not normally distributed, nonparametric tests were employed. Comparisons between groups with continuous variables were analyzed with the Kruskal-Wallis test. If there was an overall significant difference, pairwise comparisons between groups were made with the Mann-Whitney *U*-test. For categoric variables, the chi-square test was used for an overall comparison, while Fisher's exact test was used for comparisons between individual groups. The degree of association between specific IgE and IgG values was tested with the Spearman rank correlation test. A probability level of <0.05 was considered significant in all tests.

Ethics

The study was approved by the Human Research Ethics Committee at the University Hospital in Linköping, Sweden. Informed consent was given by the parents.

Results

The cumulative incidence of atopic symptoms at 9 months and the cumulative incidence of atopic diseases at 18 months of age are shown in Table 1. There were no significant differences between the feeding groups.

The lowest levels of IgG antibodies to β -lactoglobulin among the infants were seen in the N group, followed by the PH group ($P<0.01$), while the highest were seen in infants receiving RM ($P<0.001$ vs both N and PH) (Fig. 1A and B). No IgG antibodies to β -lactoglobulin could be detected in seven of the infants in the N group and in two in the PH group.

Twenty-five infants (27%) had detectable IgE antibodies to cow's milk (IgE-CM) (Table 2), and this was more common in the RM group than in either of the other two groups ($P<0.001$, Fisher's exact test). Similarly, IgE antibodies to β -lactoglobulin (IgE-BLG) were seen in 22 infants; i.e., three in the PH group and 19 in the RM group (N and PH vs RM, $P<0.001$). Five infants (5%) had detectable IgE antibodies to casein, four of them in the RM group and one in the PH group (NS). All but one of the infants with IgE antibodies to casein also had detectable IgE antibodies to β -lactoglobulin. The number of infants with detectable IgE antibodies to either of the two cow's milk proteins was significantly lower in the N and PH groups than the RM group ($P<0.001$).

There was a strong association between the levels of IgE antibodies to β -lactoglobulin and cow's milk at 9 months ($r_s=0.85$, $P<0.001$), and only 5/25 infants with IgE antibodies to cow's milk lacked IgE antibodies to β -lactoglobulin. Weaker positive correlations were seen between the levels of IgG to β -lactoglobulin on one hand and IgE to cow's milk ($r_s=0.58$, $P<0.001$) and β -lactoglobulin ($r_s=0.57$, $P<0.001$) on the other hand.

The levels of IgE antibodies to cow's milk and β -lactoglobulin were significantly higher in those infants who had a diagnosis of atopy at 9 and 18 months than in those who were healthy ($P<0.05$ and <0.01 , respectively; Mann-Whitney *U*-test), independently of the type of feeding. A similar pattern was seen for IgG antibodies to β -lactoglobulin ($P<0.05$ and 0.01) (Table 3). The difference was even more pronounced when only the diagnoses of atopic dermatitis and gastrointestinal allergy were considered. Twenty of the 25 infants who had IgE antibodies to cow's milk (range 1.1–6.5 SU/ml) did have a diagnosis of atopy up to 18 months of age.

Open or blinded oral challenges with cow's milk had been performed on clinical suspicion of cow's milk allergy in 11 infants, among them six of the 25 infants who were positive

to cow's milk in the Magic Lite system. All challenges were done after 9 months of age. Three of them were positive, supporting the diagnosis, while three of them were negative. The IgE antibody levels to cow's milk in the three challenge-positive infants were 2.8, 2.9, and 6.1 SU/ml, and 1.1, 1.6, and 1.8 SU/ml in the three challenge-negative infants.

Discussion

The lowest levels of IgG and IgE antibody responses to milk proteins were seen in the infants fed the eHF (Nutramigen), while the strongest responses were recorded in the infants fed the regular formula. In the pHF group, the levels of IgG antibody response to β -lactoglobulin were higher than in the infants fed eHF, but lower than in the regular formula group. The IgE antibody levels to cow's milk proteins were similar in the two hydrolysate groups, although IgE antibodies to β -lactoglobulin or casein were found only in the infants fed pHF or RM. However, as the study groups comprised only 29–34 infants, even a considerable difference between the pHF and N groups would not have been identified. Our results support the position statement by the European Society of Pediatric Allergy and Clinical Immunology (1) that eHF, but not pHF, should be used in the treatment of cow's milk allergy, and also support the use of extensively, and not partially, hydrolyzed cow's milk-based formulas in prophylaxis programs designed to prevent cow's milk allergy in infants.

The same partly hydrolyzed whey-casein formula as used in this study has previously been shown to induce lower IgG responses than a regular formula in infants with no defined allergy risk (21). However, the levels were significantly higher than in fully breast-fed infants. No difference in the levels of IgE antibodies to cow's milk was found between the feeding groups.

Oral introduction of cow's milk protein in early infancy elicits both cellular and humoral immune responses to cow's milk antigens (22), and the strongest immune responses are recorded during the neonatal period (23). We have very recently observed that exposure to cow's milk during the first 3 months of life is associated with high levels of IgG subclass antibodies to β -lactoglobulin at 6 and 18 months and even at 8 years of age (24), indicating a long-term effect of early antigen exposure. The higher IgG antibody response to β -lactoglobulin in infants receiving pHF than those receiving eHF prove a higher immunogenicity in the former.

An unexpectedly large number of children (21/32) in the group fed the ordinary formula had detectable IgE antibodies

to cow's milk with the Magic Lite method. In the prophylaxis study (17), only four of the same 32 children were sensitized to whole cow's milk at 9 months, as determined by CAP-RAST, while one child each was sensitized to cow's milk in the N and PH groups (NS). If the methods are measuring the same specificity, the results could indicate a higher sensitivity of the Magic Lite method to detect low levels of specific IgE. Another possibility would be *in vitro* cross-reactivity with other proteins, as has been described for human milk proteins and bovine cow's milk proteins (25). However, low levels of IgE antibodies to food antigens may reflect the normal immune response and are not always associated with clinical allergy (26). In our study, 6/25 cow's milk-positive infants had levels between 1.0 and 1.43 SU/ml, which, according to the manufacturer, corresponds to values below class 1 in the RAST system. Only one of them was healthy at 18 months, however, and, in all, 20 of the 25 infants with detectable IgE antibodies to cow's milk had an atopy diagnosis at the same age. Although occasional intake of cow's milk products outside the feeding regimen could explain some of the sensitization, we believe that the results reflect the immunogenicity of the formulas.

To support a claim for allergy prevention, infants with a family history of allergy should be fed formulas in controlled, randomized studies for at least 6 months with careful monitoring of clinical outcome, and reactions to the formulas must be evaluated in double-blind, placebo-controlled oral challenge (4). It seems valuable to add monitoring of IgE and IgG antibodies to cow's milk proteins with sensitive methods during the feeding period, as these might reveal an immunogenicity of the formulas not easily discovered in the clinical evaluation. Our study indicates that atopic infants are particularly likely to have IgE and IgG antibody responses to cow's milk proteins.

In conclusion, the extensively hydrolyzed cow's milk-based formula seemed less likely to elicit an immune response during a feeding period than the partially hydrolyzed and nonhydrolyzed cow's milk-based formulas, thus supporting the use of extensive hydrolysates, but not partial hydrolysates, for prophylactic treatment of infants at high risk of subsequent allergy.

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