

Markers of inflammation in the feces of infants with cow's milk allergy

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Growing evidence exists that exposure to cow's milk elicits inflammation in the gut of infants with cow's milk allergy, irrespective of symptoms. To demonstrate inflammation and increased protein leakage from the gut during a cow's milk elimination-challenge test in fecal samples of infants presenting with different symptoms suggestive of cow's milk allergy, we measured the concentrations of α_1 -antitrypsin (AT), eosinophil cationic protein (ECP), immunoglobulin (Ig) A, and cow's milk-specific IgA antibodies, in fecal samples of 208 infants with a mean age of 7 months. Prechallenge samples were collected after a mean 3-week elimination period, and post-challenge samples were obtained 4 days after starting the challenge. Fecal levels of prechallenge total IgA ($p = 0.02$) and post-challenge AT ($p = 0.001$) were higher in infants with a positive challenge. Of these infants, pre- and post-challenge levels of ECP were higher in those reacting after 24 h than in those reacting within 1 h ($p = 0.006$ and $p = 0.045$). Prechallenge levels of ECP were higher in those showing intestinal symptoms ($p = 0.008$), and both pre- and post-challenge levels of total IgA were higher in those with an IgE-mediated reaction to cow's milk ($p = 0.04$ and $p = 0.008$). Regardless of the challenge result, total IgA increased during the challenge ($p < 0.001$ for both challenge-positive and -negative infants) and was higher in those breast-fed until the challenge than in those fed formula only ($p < 0.01$). Hence, in infants reacting to the cow's milk challenge, higher prechallenge levels of fecal IgA indicate increased antigenic stimuli in the gut, and higher post-challenge levels of AT reflect increased protein loss as a result of intestinal inflammation. In infants with slowly evolving gastrointestinal symptoms, increased fecal ECP may help in distinguishing patients from those who tolerate cow's milk. Individual serial follow-up of fecal IgA and ECP can be used to estimate the degree of inflammation in the gut and an appropriate time for a challenge test, but are not diagnostic tools for cow's milk allergy.

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A prerequisite for sensitization to food is the passage of antigenic material through the gut mucosa (1). The permeability of the gut is highest immediately after birth and diminishes with the maturation of specific and non-specific defence factors of the gut (2,3). However, viral infections of the intestine (4) and other types of inflammation, such as celiac disease and inflammatory bowel disease (5), may also enhance mucosal permeability. Food allergy is often associated with specific immunoglobulin (Ig)E antibodies, but in a large proportion of patients, other

mechanisms, such as secretion of tumour necrosis factor- α (TNF- α), are involved (6,7). Patients with food allergy differ from each other by symptoms, which most typically evolve from the skin, and gastrointestinal and respiratory tracts (8). Symptoms may depend on the location and quantity of sensitized mast cells (9). When the gut matures, its permeability to food antigens is lost and, even with continued IgE reactivity to food antigens, clinical sensitivity is lost (10).

We sought markers of inflammation and increased leakage of the gut to further our under-

standing of the immune reactions in cow's milk allergy (CMA). In addition, we evaluated the possible diagnostic value of these markers in CMA. We studied a large, unselected group of infants with CMA, showing different types of symptoms, and compared them to a group of infants not reacting in a cow's milk (CM) challenge test (11,12). In the group of 239 infants challenged with CM, we studied the fecal content of α_1 -antitrypsin (AT), eosinophil cationic protein (ECP), IgA, and CM-specific IgA antibodies, and evaluated the association of these measurements with clinical reactivity to CM and with the type of allergic reaction that the infants with a positive CM challenge presented. The influence of the type of milk (breast or formula) on the fecal markers was also studied.

Subjects and methods

Subjects

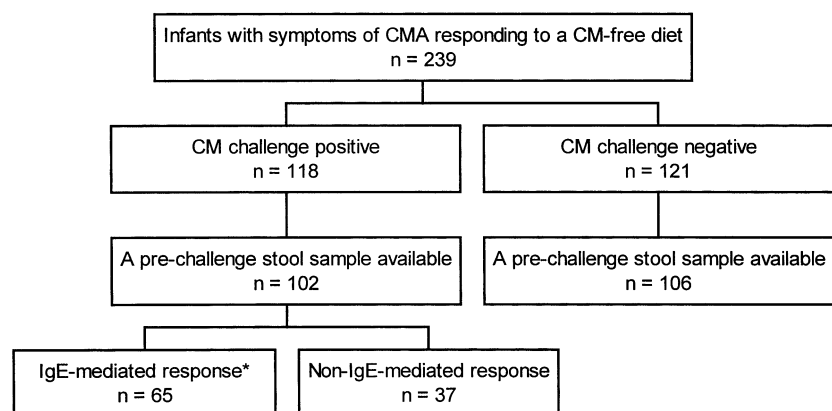
The patients with CMA had been diagnosed in a study on the effects of different types of supplementary milk given in delivery hospitals on the subsequent incidence of CMA (11). Of the 6,209 participating infants, 239 were suspected to have CMA on the basis of symptoms and the disappearance of these symptoms during a CM-free diet. After a successful elimination lasting from 2 to 4 weeks, the challenge was administered to these infants at a mean age of 7 months. The CM challenge was carried out at the out-patient clinic, supervised by one of the authors (K.M.S.) of the present report. CM formula was given in quantities of 1, 10, 50, and 100 ml at intervals of 30–60 min. The next day, infants without symptoms continued to receive the formula at home with doses appropriate for age. K.M.S. examined all patients on day 5, or earlier if requested, to check for delayed symptoms. The test was considered

positive if one or more of the following symptoms had appeared in the interim: urticaria, exanthema, atopic dermatitis, vomiting, diarrhea, wheezing, allergic rhinitis, or conjunctivitis. Infants with an adverse reaction to the challenge continued on a CM-free diet and those without symptoms continued on the formula. One month later, K.M.S. examined all patients to confirm the challenge result. However, no clinical relapses occurred between the challenge and the control visit.

Patients were categorized into IgE- and non-IgE-mediated groups according to the presence or absence of CM-specific IgE antibodies, respectively (12). Those with an IgE-mediated reaction had a skin-prick test of ≥ 3 mm and/or IgE CM antibodies of ≥ 0.7 kU/l. In those with a non-IgE-mediated reaction, both tests were negative. The skin-prick test was performed before the CM challenge with liquid CM formula, according to a standard technique (13). Histamine dihydrochloride 10 mg/ml served as a positive control and 0.9% saline solution was used as a negative control. IgE CM antibodies were measured by using the Pharmacia CAP system (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden).

A prechallenge stool sample was available for 102 of the 118 challenge-positive and for 106 of the 121 challenge-negative infants (Fig. 1). A post-challenge stool sample was available for 98 and 102 infants, respectively. Of the challenge-positive infants, 65 showed an IgE-mediated and 37 a non-IgE-mediated reaction (Fig. 1). During the challenge, 37 infants showed clinical symptoms within 1 h, 40 between 1 and 24 h, and the remaining 25 within 4 days from the beginning of the challenge. During the challenge test 93 infants showed dermatologic signs (51 urticaria/exanthema, 46 deterioration of eczema), 22 had gastrointestinal symptoms, 11 had respiratory symptoms, and five had eye symptoms; 26 had

Fig. 1. Flow chart of study subjects according to their response to a cow's milk (CM) elimination-challenge test and the presence or absence of immunoglobulin E (IgE) CM antibodies. CMA, cow's milk allergy. The asterisk refers to a skin-prick test of ≥ 3 mm and/or a serum IgE CM antibody level of ≥ 0.7 kU/l.



symptoms from several organs, one of them an anaphylactic reaction.

Fecal samples

A prechallenge stool sample was taken at home in a vial within 12 h of the forthcoming visit and stored in a refrigerator. The wet sample was weighed and lyophilized. The material was dissolved in a volume of phosphate-buffered saline (PBS) that was five times the weight of the original stool sample. The sample was then shaken for 1 h, centrifuged at high speed and the clear supernatant was stored at -20°C for subsequent analysis. Four days after commencement of the CM challenge (and irrespective of the challenge outcome), the post-challenge sample was collected and processed identically to the prechallenge sample.

Methods

The fecal concentrations of total and specific IgA were measured to estimate intestinal maturity and degree of local stimulation. Total levels of IgA in fecal extracts were measured by using an enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc-Immuno Plates; Nunc, Roskilde, Denmark) were coated overnight at $+4^{\circ}\text{C}$ with anti-IgA antibodies (Dako, Glostrup, Denmark), diluted to 1:1000 in 50 mM NaHCO_3 , pH 9.5 (100 μl /well). The coated plates were washed once with PBS (0.02 M Tris, 0.5 M NaCl, pH 7.5), saturated for 1 h at room temperature with PBS containing 2% bovine serum albumin (BSA), and washed again with PBS. Standards (Serum Protein Calibrator; Orion Diagnostica, Espoo, Finland) and fecal extracts, diluted (typically 1:10 000) in diluting buffer (PBS containing 1% BSA and 0.05% Tween-20), were distributed to microtiter plates (100 μl /well) and incubated overnight at room temperature. The plates were washed three times with PBS containing 0.05% Tween-20. Alkaline phosphatase-conjugated anti-human IgA (Dako), diluted to 1:1000 in diluting buffer, was added and the plates were developed as described for the CM-antibody assay (14). The detection limit of the total IgA assay was 5 μg of IgA/l of the diluted fecal extract. For CM-antibody ELISA, the microtiter plates were coated with a diluted, adapted CM formula (Tutteli; Valio, Helsinki, Finland). Results were compared to a serum sample with a high titre of antibodies and expressed as a percentage in relation to the reference serum. As a marker of protein leakage into the gut, fecal AT was measured by using single radial immunodiffusion. The antiserum (Orion Diagnostica) was diluted to

1:37.5 in the agar. The standard was Serum Protein Calibrator (Orion Diagnostica). Diffusion was allowed to occur for 1 week at $+4^{\circ}\text{C}$. ECP was measured to demonstrate local eosinophil degranulation in the gut by using the Pharmacia CAP system (Pharmacia & Upjohn Diagnostics).

Statistical analyses

The distributions of continuous data were skewed. Thus, all comparisons were made after logarithmic transformation. Analysis of variance (anova) was used for multiple comparisons and the Student's *t*-test for comparisons between two groups. For comparisons between the paired measurements, we used a paired *t*-test. The data are presented with geometric means and 95% confidence intervals (CI), or with medians and ranges. For categorical variables, we used Pearson's chi-square test. Two-sided tests of significance were used throughout. Statistical significance was defined as a *p*-value of ≤ 0.05 . Analyses were performed using an SPSS (version 7.5 for Windows; SPSS, Chicago, IL, USA) software package.

Ethics

The ethics committee of the Hospital for Children and Adolescents approved the study protocol. Written, informed consent was obtained from the mother of each participating infant.

Results

Positive and negative challenges and fecal ECP, AT, and IgA

The infants with a positive CM challenge had a higher prechallenge level of fecal total IgA than those with a negative challenge (Table 1). Similarly, the prechallenge level of ECP tended to be higher in infants with CMA. The post-challenge level of AT was elevated more in infants with a positive challenge than in those with a negative challenge. The fecal IgA increased significantly during the challenge both in infants with CMA and in those not showing symptoms at challenge (Table 1). Fecal IgA antibodies to CM could be measured in 44/88 (50%) and 38/96 (40%) of post-challenge samples of infants with positive and negative challenges, respectively, the levels being similar in both groups (data not shown).

Fecal ECP, AT, and IgA and infants with a positive challenge

We grouped and compared the infants with a positive challenge in three different ways, as follows: infants with and without gastrointestinal

Table 1. Geometric means [95% confidence interval (CI)] for pre- and post-challenge fecal immunoglobulin (Ig)A, α_1 -anti-trypsin (AT), and eosinophil cationic protein (ECP) in infants with positive and negative responses to cow's milk (CM) challenge

Fecal measurement	Infants with a positive challenge		Infants with a negative challenge		p-value (t-test)
	n	Mean (95% CI)	n	Mean (95% CI)	
IgA, g/l					
Prechallenge	100	0.55 (0.48–0.64)*	106	0.44 (0.39–0.50)*	0.02
Post-challenge	96	0.66 (0.58–0.76)*	99	0.59 (0.52–0.67)*	0.23
AT, g/l					
Prechallenge	92	0.12 (0.06–0.24)	104	0.07 (0.04–0.14)	0.24
Post-challenge	90	0.18 (0.10–0.30)	99	0.03 (0.02–0.07)	0.001
ECP, μ g/l					
Prechallenge	102	68.0 (53.7–86.0)	104	51.3 (41.2–63.8)	0.09
Post-challenge	98	59.4 (47.5–74.3)	102	58.9 (47.4–73.3)	0.96

*p < 0.001 between pre- and post-challenge values, paired t-test.

symptoms (diarrhoea and/or vomiting) (7); those with rapid, intermediate or slow onset of symptoms (15,16); and those with and without evidence of IgE-mediated reaction to CM (16,17). Both pre- and post-challenge levels of ECP were higher in infants showing intestinal symptoms at challenge, the difference being significant between the prechallenge values (Table 2). Furthermore, the prechallenge level of ECP in infants with intestinal symptoms differed significantly from that of tolerant infants (p = 0.002). When the infants reacting within 1 h to a small dose of CM formula (median 1 ml, range: 1–11 ml) were compared with those reacting after 24 h to large volumes of CM (median 1600 ml, range: 200–3400 ml), the infants who reacted more slowly had higher levels of both pre- and post-challenge fecal ECP (Table 2). Pre- and post-challenge levels of fecal IgA and AT were similar in infants with different symptoms and in those grouped according to the onset of reaction (data not shown). The infants with an IgE-mediated reaction to CM had significantly higher levels of both pre- and post-challenge fecal IgA (Table 3). The fecal IgA also increased significantly during the challenge among these infants. Fecal levels of CM-specific IgA antibodies did not differ

between any of the above sub-groups (data not shown).

Type of milk fed and fecal IgA

Breast-feeding had been ceased in 38 (37%) infants with a positive and in 58 (56%) infants with a negative challenge (p=0.008) at mean 1.8 (95%CI 1.2–2.3) and 2.9 (95%CI 2.2–3.5) months before the challenge (p=0.02), respectively. The mean total duration of the CM elimination period was 27 days in breast-fed infants and 24 days in those formula-fed before the challenge. Both pre- and post-challenge levels of fecal IgA were higher in infants breast-fed than in those formula-fed before the challenge; the difference was similarly significant in infants with and without CMA (Table 4). Among breast-fed infants, the prechallenge IgA was significantly higher in those with CMA than in those with a negative challenge. The level of fecal IgA increased during the challenge in all infants irrespective of the feeding group or the challenge result, the increase being statistically significant in the formula-fed infants with a negative challenge. The levels of fecal ECP and AT were not influenced by the type of milk fed (data not shown).

Table 2. Geometric means [95% confidence interval (CI)] of pre- and post-challenge fecal eosinophil cationic protein (ECP) levels in infants with cow's milk allergy (CMA) in relation to reaction time and symptoms at challenge

Infants with CMA	Prechallenge fecal ECP (μ g/l)			Post-challenge fecal ECP (μ g/l)		
	n	Mean (95% CI)	p-value	n	Mean (95% CI)	p-value
Symptoms at challenge						
Intestinal	22	124.0 (70.1–220)	0.008	21	75.8 (48.0–119)	0.27
No intestinal symptoms	80	57.6 (45.1–73.6)		77	55.6 (43.1–71.7)	
Reaction time at challenge						
<1 h	37	50.8 (36.6–70.4)*	0.02	35	46.4 (32.6–66.0)†	0.12
1–24 h	40	63.1 (43.2–92.1)		40	59.5 (42.2–83.7)	
>24 h	25	118.0 (70.2–198)*		23	86.3 (52.6–142)†	

*p = 0.006, †p = 0.045 between infants with rapid and slow reactions to cow's milk (CM) challenge, t-test.

Table 3. Geometric means [95% confidence interval (CI)] for pre- and post-challenge fecal immunoglobulin (Ig)A, α_1 -antitrypsin (AT) and eosinophil cationic protein (ECP) in cow's milk (CM)-allergic infants with IgE- and non-IgE-mediated reaction to CM measured at the time of challenge

Fecal measurement	IgE-mediated reaction		Non-IgE-mediated reaction		p-value (t-test)
	n	Mean (95% CI)	n	Mean (95% CI)	
IgA, g/l					
Prechallenge	64	0.62 (0.52–0.74)*	38	0.45 (0.36–0.58)	0.04
Post-challenge	61	0.76 (0.65–0.89)*	35	0.52 (0.41–0.66)	0.008
AT, g/l					
Prechallenge	57	0.12 (0.05–0.27)	35	0.13 (0.04–0.39)	0.92
Post-challenge	57	0.14 (0.07–0.30)	32	0.26 (0.12–0.56)	0.33
ECP, μ g/l					
Prechallenge	65	62.4 (46.6–83.6)	37	78.9 (53.0–117)	0.35
Post-challenge	61	57.5 (42.7–77.4)	37	62.7 (44.9–87.7)	0.71

*p = 0.04 between pre- and post-challenge values, paired t-test.

Discussion

We analyzed the fecal concentrations of total and CM-specific IgA as markers of maturity and degree of stimulation of the local immune system of the gut (18), ECP as a marker of eosinophil degranulation in the intestine (9), and AT as a sign of protein loss as a result of increased inflammation in the intestine (19). We compared levels of these measurements between infants who had either developed symptoms during the CM challenge or remained clinically unresponsive. The tolerant infants were also suspected of CMA, which probably influenced results. They had experienced similar symptoms to those with a positive challenge, and they had become asymptomatic during a CM-free diet (11,12). Although these infants did not react clinically to the challenge, some showed a positive skin-prick test or had increased levels of CM-specific IgE antibodies (20). In all fecal measurements taken either before or after the challenge, we found some differences between the infants with positive and negative challenges, but the differences were small and the overlap between the groups was considerable. Higher prechallenge IgA and elevated post-challenge AT in infants with CMA, and higher prechallenge ECP in allergic infants with intestinal symptoms, suggest that more

inflammation, protein loss, and antigenic stimuli occur in the intestines of infants with CMA. Nevertheless, many allergic infants had similar levels of markers as those clinically tolerating CM.

The CM-allergic infants showing intestinal symptoms during the challenge had higher levels of fecal ECP than those with other symptoms or those tolerating CM. This suggests that the local release of eosinophilic material in the intestine is important in the appearance of gastrointestinal symptoms of CMA. Similarly, increased levels of ECP have been measured locally in nasal washes of wheezing infants as a marker of airway inflammation (21). When compared with serum levels, the levels of ECP found in feces (19) or in different body fluids (21,22) have more accurately correlated with the clinical allergic symptoms. In our study, the level of ECP was higher in infants with a slow onset of symptoms than in those with rapid reaction at challenge. Previously we found that both infants reacting slowly and those showing gastrointestinal symptoms at challenge more often had an IgE-negative response to CM (12). In these patients, the allergic inflammation of the intestine is probably caused by mediators other than IgE, with eosinophils presumably playing an impor-

Table 4. Geometric means [95% confidence interval (CI)] for pre- and post-challenge levels of fecal immunoglobulin (Ig)A in relation to infant-feeding regimen prior to cow's milk (CM) challenge in infants with positive and negative responses to challenge

Type of feeding prior to challenge	Prechallenge fecal IgA (g/l)		Post-challenge fecal IgA (g/l)		p-value (t-test)
	n	Mean (95% CI)	n	Mean (95% CI)	
Infants with positive challenge					
Breast milk*	63	0.72 (0.61–0.84)†	63	0.79 (0.69–0.91)	0.12
No breast milk*	39	0.36 (0.30–0.45)	35	0.48 (0.37–0.62)	0.06
Infants with negative challenge					
Breast milk*	45	0.54 (0.44–0.67)†	49	0.73 (0.60–0.88)	0.09
No breast milk*	61	0.38 (0.33–0.44)	50	0.49 (0.43–0.55)	<0.001

*p < 0.01 for both pre- and post-challenge levels of IgA between infants breast-fed and exclusively formula-fed prior to challenge.

†p = 0.04 between challenge-positive and -negative breast-fed infants.

tant role (9). Therefore, measurement of fecal ECP may help in discriminating between hyper-sensitive and tolerant infants in these sub-groups of patients. We could not, however, detect any increase in fecal ECP during the challenge. We probably collected the post-challenge samples too late to observe the effect of the challenge on eosinophil activation and the ensuing release of ECP. In a previous study, the introduction of CM to gut lumen caused a local rise in ECP within 40 min in sensitized patients and levels were normalized within 2 h (23). The prechallenge levels of fecal AT were similar in allergic and tolerant infants. However, the post-challenge level was significantly higher in infants with CMA. This suggests that in reactive infants, irrespective of symptoms, the CM challenge induces intestinal inflammation which increases gut permeability and protein loss. This altered permeability can be measured up to several days after the challenge (24).

The mean duration of the CM-elimination diet before the challenge was slightly longer than 3 weeks. The symptoms started to subside quite rapidly, on average 5 days after commencement of the elimination diet (12). However, that the challenge affected only total IgA levels and that the infants with slow-appearing symptoms had significantly higher pre- than post-challenge levels of ECP suggest that the period of CM elimination before the challenge should be longer to reduce the inflammation in the intestine more fully.

A rise in fecal IgA was also seen in infants not reacting clinically to the CM challenge, suggesting that such a reaction is indicative of the diet change to more antigenic and may be protective. Alternatively, a strong local secretion of IgA is seen in untreated celiac disease, in which intestinal inflammation is caused by dietary gluten (25). Both pre- and post-challenge levels of fecal IgA were higher in infants breast-fed until the challenge. Passage of maternal IgA through the infant's gastrointestinal tract has been detected (26). However, the presence of maternal IgA does not explain the prechallenge level of fecal IgA being higher in breast-fed infants with a positive challenge than in those tolerating CM. Breast milk contains small amounts of CM proteins (27), which are capable of triggering immune responses in infants (28). We suggest that in the infants with CMA even the small amounts of CM proteins in breast milk enhance the intestinal IgA response. The increased level of IgA during the challenge was of similar magnitude in the breast- and formula-fed infants, further suggesting that the change in the IgA level is a result of the infants' own responses.

The present study shows that many infants who react to the CM challenge show increased levels of IgA in feces as a marker of increased antigenic stimuli in the gut. They also lose a greater quantity of plasma proteins to the intestinal lumen and have a higher level of eosinophilic proteins, such as ECP, in feces than infants who tolerate CM. While the marked overlap of the groups does not allow the use of these tests as diagnostic tools, individual serial follow-up of fecal IgA and ECP (29) may be used to estimate the degree of inflammation of the intestine and to determine appropriate times for the challenge test, which should not be performed as long as markers of inflammation are at a high level in the feces.

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