

A similar high level of immunoglobulin A and immunoglobulin G class milk antibodies and increment of local lymphoid tissue on the duodenal mucosa in subjects with cow's milk allergy and recurrent abdominal pains

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In previous studies, we have reported endoscopic and histological alterations locally on the gastrointestinal (GI) tract associated with a gastrointestinal type of cow's milk allergy. In this study, we sought to further characterize endoscopic, and immunological findings in these children. We also hypothesized that the same type of immune responses might also be found in children with unexplained and recurrent abdominal pains. We did a gastroduodenoscopy for persistent GI symptoms, examined the mucosal histology of the small intestine and measured the antibodies to whole cow's milk and its fractions with an enzyme-linked immunosorbent assay (ELISA) in a consecutive series of 22 subjects with untreated and 14 with treated cow's milk allergy (CMA) and 44 with recurrent abdominal pains (RAP). The immunological findings of the study subjects were compared with 54 controls.

Lymphonodular hyperplasia (LNH) of the duodenum was the main endoscopic finding in 11 subjects (50%) with untreated and 5 (36%) with treated CMA. It was also found in 6 of 44 subjects with RAP. Compared with the controls, the patients with CMA showed significantly higher levels of IgA class antibodies to whole milk ($p = 0.003$) and β LG ($p < 0.0001$). Of the IgG class antibodies to β LG ($p = 0.032$), BSA ($p < 0.0001$) and α CAS ($p < 0.0001$) were significantly higher. The patients with LNH of the duodenal bulb as the main endoscopic finding showed significantly higher values of IgG class antibodies to β LG ($p = 0.01$) and α CAS ($p = 0.005$). Interestingly, the patients examined for RAP showed a similar increment in the pattern of whole milk and specific milk protein antibodies as the CMA children. In conclusion this study showed that gastrointestinal CMA beyond infancy is significantly associated with high levels of IgG and IgA class antibodies to milk and its fractions. As high levels of these antibodies and LNH of the duodenal bulb were also found in subjects with RAP, the study further suggests that gastrointestinal CMA might be one major reason for RAP.

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The mucosal lesions of the gastrointestinal tract associated with cow's milk allergy (CMA) vary from villous atrophy to completely normal morphology and manifest as a patchy appearance

at any site of the gastrointestinal (GI) tract (1,2). The time, duration and quantity of oral antigen exposure also contribute to the immune reaction of the intestinal mucosa. Moreover, increased

mucosal permeability and, consequently, influx of antigenic material into the mucosa and the host response based on the tolerance finally determine the intensity and nature of the reaction. The propensity to immunological tolerance is genetically determined, and different mechanisms may intervene with the mucosal reactions (3,4).

In addition to the well-established immunological lesions of intestinal mucosa, *viz.* the eosinophilic reaction and celiac-like mononuclear infiltration, we recently described in school-aged children with delayed CMA patchy lymphonodular hyperplasia (LNH) of the GI tract as the most common lesion. It was found in endoscopies of two-thirds of the patients with untreated CMA, and it was most prominent on the duodenal bulb (5,6).

Previous cross-sectional and prospective studies have shown that CMA of infancy is associated with elevated levels of immunoglobulin G (IgG)1 and IgG4 subclass antibodies to cow's milk proteins (7,8). In our children with delayed CMA who reacted within a few days after starting the challenge with increasing doses, the histological finding in duodenal biopsy was totally different from that seen in infants with cow's milk protein intolerance. They typically show abundant lymphoid nodules with germinal centers without diffuse mononuclear or eosinophilic cell infiltration. We hypothesized that this delayed CMA beyond infancy might be caused by IgG class antibodies against cow's milk proteins. To test this hypothesis, we determined IgA and IgG antibodies to whole cow's milk and its specific proteins in 22 untreated cases diagnosed with an open elimination-challenge test for CMA and in 14 subjects who had been diagnosed for CMA earlier and were on a milk elimination diet. The results were compared with 44 subjects examined for recurrent abdominal pains (RAP) and without obvious signs of CMA and 54 randomly selected school-aged children. All the subjects except the healthy controls were scheduled for gastroduodenoscopy.

Patients and methods

Patients

This study covered a consecutive series of 22 (12 female, mean age 9.0 ± 3.4 years, range 3–15 years) children with untreated food CMA and 14 (6 female, mean age 7.8 ± 4.3 years, range 3–11 years) children with treated CMA, who were on an elimination diet at the time of the study. The subjects had been referred, during a period of 3 years, for pediatric gastroenterologi-

cal consultation in the Oulu University Hospital because of persistent intestinal symptoms or growth retardation indicating a need for gastroduodenoscopy. The parents filled in a questionnaire for the registration of sociodemographic data, gastrointestinal and atopic symptoms and manifestations (atopic dermatitis, allergic rhinitis and asthma), the history of suspected and proven food allergy and the method of diagnosing it, and other gastrointestinal symptoms (vomiting, nausea, abdominal pains as well as the frequency and characteristics of the stools) of their child.

Most patients had never been seen in our hospital before this referral. After the first symptoms of suspected CMA, the subjects were scheduled for oral challenges with milk, continued for 2 weeks if the symptoms did not manifest earlier (9).

The findings of these two CMA groups were compared with 44 (26 females, mean age 9.9 ± 3.4 years, range 3–15 years) children with recurrent abdominal pains (RAP) lasting for at least 3 months but examined with gastroduodenoscopy without any suspicion of CMA and 54 (25 females, mean age 11.1 ± 1.5 years, range 9–11 years) children selected at random from local schools and examined as a control group for our more comprehensive study on the outcome of milk allergy in infancy.

Diagnosis of food allergy

Fourteen subjects had been diagnosed for CMA before enrollment in the present study and were on a milk-free diet. The subjects suspected to have CMA were scheduled for a 2-week milk elimination diet followed by a 2-week oral challenge with low-lactose milk. Abdominal pains, diarrhea and loose mucous stools, and exacerbation of atopic dermatitis were considered as a positive result. Lactose intolerance was considered as a separate entity and diagnosed with a lactose load test in patients reporting bouts of abdominal pains, diarrhea and/or flatulence during a lactose-containing diet.

The diagnosis of atopic manifestations (atopic dermatitis, asthma, allergic rhinitis) was based on general criteria and was only accepted if made by a doctor. As evident in Table 1, the study subjects had had more atopic manifestations than the controls.

The main indication for an endoscopic examination in the subjects with untreated CMA were abdominal pains in 13, diarrhea in 8 and anemia in 1. Eight subjects with treated CMA were studied for abdominal pains, two for intractable diarrhea and four for growth retardation.

Table 1. Clinical, endoscopic and histological findings of children studied for IgA and IgG class antibodies to whole cow's milk and its specific proteins [n (%)]

	CMA, untreated	CMA, treated	RAP	Controls
n	22	14	44	54
Females	12	6	26	25
Mean age	9.0±3.4	7.8±4.3	9.9±3.9	11.1±1.5
Atopic manifestations				
Dermatitis	13 (59)	8 (57)	8 (18)	10 (19)
Asthma/rhinitis	5 (23)	5 (36)	4 (9)	2 (4)
Endoscopic findings				
Esophagitis	2 (9)	0	3 (7)	—
Gastritis/erosions	2 (9)	5 (36)	10 (23)	—
Duodenitis, lymphonodular	11 (50)	5 (36)	6 (14)	—
Erosive	1 (4)	2 (14)	2 (5)	—
In a duodenal biopsy increased number of				
eosinophils	9 (41)	3 (21)	2 (5)	—
mononuclear cells	2 (9)	2 (14)	1 (2)	—

Endoscopic examinations, biopsy specimen sampling and processing

Gastroduodenoscopy was performed with an Olympus GIF-JT140 gastroscope (Olympus, Tokyo, Japan) under general anesthesia, as usual in our hospital. Biopsies were taken for routine histology from the duodenum distally from the sphincter of Oddi, the antrum of the stomach and the lower part of the esophagus, each at the most demonstrative site where local pathology could be seen. Another sample for the detection of *Helicobacter pylori* was taken from the stomach. Patients with findings indicative of celiac disease were excluded.

Histopathology

All biopsy specimens were re-evaluated by one pathologist (TK) blinded to the clinical or endoscopic data. The biopsy specimens were fixed in 5% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. The gastric and duodenal biopsies were evaluated in terms of the established criteria. The content of eosinophilic leukocytes was graded as follows: grade 0, no eosinophils; grade 1, 1–5 per high-power field (40×obj); grade 2, 6–10/high-power field; grade 3, >10/high-power field.

Determination of cow's milk antibodies

Cow's milk antibodies of IgA and IgG isotypes and IgG and IgA antibodies to bovine β -lactoglobulin (β LG), bovine serum albumin (BSA) and α -casein (α CAS) were measured with an enzyme-linked immunosorbent assay (ELISA) as described earlier with modifications (1–3). Microtiter plates (MaxiSorp®, Nunc A/S, Roskilde, Denmark) were coated with the following antigens: adapted liquid CM formula (Tutteli®, Valio Ltd, Helsinki, Finland) defatted

and diluted (1:500 in carbonate buffer, pH 9.6), bovine β LG (Sigma Pharmaceuticals, St. Louis, USA, at a concentration of 1 μ g/ml in carbonate buffer, pH 9.6), BSA (grade V, Sigma) and α -casein [Sigma, in concentrations of 2 μ g/ml in phosphate-buffered saline (PBS), pH 7.4]. All were incubated overnight. Wells were saturated either with 0.5% sheep serum (for anti-CM and β LG assays) or with 1% gelatin in PBS pH 7.4 (for BSA and α -casein assays). Serum samples were diluted in the same buffers as used in the saturation of wells. Triplicate dilutions for assays were 1:20 for β LG, 1:40 for cow's milk and BSA and 1:100 for α CAS.

In the assays for cow's milk and β LG antibodies the plates were incubated with serum dilutions overnight at room temperature, whereas

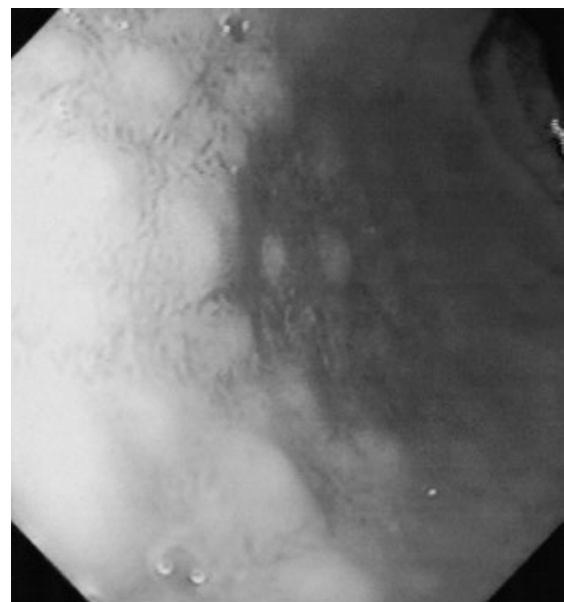


Fig. 1. Lymphonodular hyperplasia of the duodenal bulb with normal villous architecture in a 9-year-old girl with cow's milk allergy.

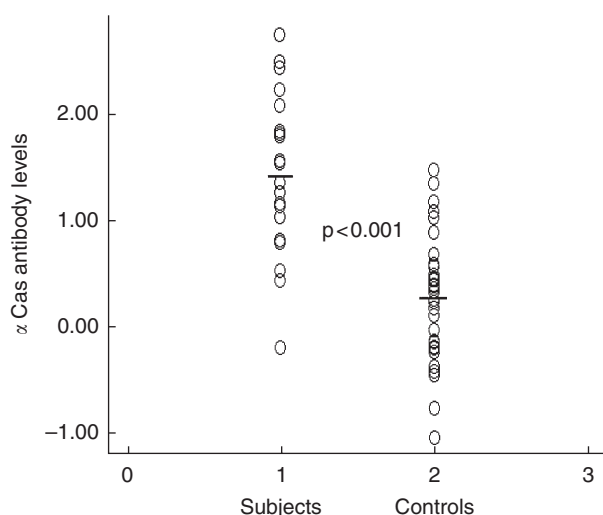


Fig. 2. The levels of α -casein (α Cas) antibodies in subjects with untreated cow's milk allergy or recurrent abdominal pains and lymphonodular hyperplasia of the duodenal bulb and in non-atopic controls.

in the assays for BSA and α CAS they were incubated at 37°C for 1 h.

After washing, 100 μ l of alkaline-phosphatase-conjugated monospecific rabbit affinity-purified F(ab')₂ antihuman IgG, IgA and IgM antisera (dilutions between 1:600 and 1:1200) (Dako A/S Glostrup, Denmark) were added, and the plates were incubated for 60 min at 37°C. After

washing, 100 μ l of p-nitrophenylphosphate substrate [2 mg/ml in diethanolamine buffer, pH 10.0 (both from Medix Biochemica, Helsinki, Finland)] was added. The reaction was stopped after 30 min with 100 μ l of 1 M NaOH. The end product was measured at 405 nm in a semiautomatic photometer (Titertek Multiscan®, Elflab Inc., Helsinki, Finland). The mean value of the two absorbances for the wells coated with blocking solution was subtracted from the mean value for the three absorbances in the antigen-coated wells. This result was compared by point-to-point analysis with the computerized photometer with the result for twofold serial dilutions of a standard serum with a very high titer of cow's milk antibodies. The value for standard serum was given as 100%. If the absorbance of the sample was not on the linear part of the standard curve, the sample was diluted further. The levels of antibodies were expressed as percentages of the standard.

Determination of serum IgE

The concentrations of IgE were determined with a chemiluminescence method and the concentrations over 360 kU/l as the upper value of age-matched controls in our hospital were considered as elevated.

Table 2. The median values (CI₉₅) of IgA and IgG class antibodies to whole cow's milk and its specific fractions in children with untreated and treated cow's milk allergy (CMA) and in children with recurrent abdominal pains (RAP) and in the controls

		CMA, untreated	CMA, diet	RAP	Controls
n		27	14	44	54
α -Casein	IgG	16.5 (2.8–62.9)	9.15 (1–68.9)	6.6 (2.7–17)	1.8 (0.9–2.8)
		<0.0001	0.006	<0.0001	
	IgA	5.3 (1.2–7.7)	0.8 (0.5–2.4)	2.6 (1.2–4.3)	3.8 (2–5.2.0)
		0.69	<0.0001	0.25	
Bovine serum albumin	IgG	211.0 (63.9–398.0)	50.9 (0–246.2)	79.2 (49.2–124.5)	34.8 (21.7–64.6)
		<0.0001	0.59	0.009	
	IgA	0.0 (ND-706.3)	0.0 (ND-12.3)	0.0 (ND-220)	0.0 (ND-784)
		0.0	0.0	0.0	0.0
β -lacto-globulin	IgG	27.4 (7.4–45.8)	12.6 (3.6–23.0)	6.4 (2.7–16.2)	6.7 (3.1–15.8)
		0.032	0.15	0.82	
	IgA	0.4 (0.2–0.6)	0.2 (0.0–0.5)	0.2 (0.1–0.4)	0.0 (0.0–0.1)
		<0.0001	0.003	<0.0001	
Whole cow's milk	IgG	45.5 (8.4–1933.0)	191.0 (4.2–1955.0)	88.5 (18.0–450.0)	9.0 (5.4–14.3)
		0.30	0.012	<0.0001	
	IgA	8.6 (3.4–38.0)	18.2 (0.4–81.8)	9.5 (3.4–73.7)	2.5 (1.46–3.51)
		0.003	<0.0001	<0.0001	

The significance is presented as compared to the controls.

Statistical analysis

The data collected from the questionnaires and the clinical findings were analyzed with the SPSS package. A 95% confidence interval was used whenever appropriate to estimate the standard error, and the Chi-squared test was used to estimate the significance of the differences between the subgroups. The CIA[®] software was used to calculate medians with CI₉₅ when processing the milk antibody results. The medians with CI₉₅ were presented as a binomial distribution due to the small number of cases. The statistical significance of the difference in milk antibody levels between the groups was calculated using the Mann–Whitney *U*-test.

Ethical considerations

Written parental consent with a signature after an oral explanation of the study plan was obtained from the parents for all children. The protocol was approved by the Ethics Committee for Clinical Science of the Oulu University Hospital.

Results

Endoscopic and histological findings

The main gastrointestinal symptoms in subjects with untreated CMA were bouts of abdominal pains in 13 subjects, prolonged diarrhea and/or loose stools in 8 and anemia with growth retardation and dermatitis in 1. Moreover, more than half of the subjects had atopic dermatitis and a quarter asthma (Table 1). Lymphonodular hyperplasia (LNH) of the duodenum, as demonstrated in Fig. 1, was the main endoscopic finding in 11 (50%) subjects with untreated and 5 (36%) with treated CMA (Table 1). Moreover, it was found in 6 of the 44 patients with RAP. Three study subjects and two RAP patients further showed erosive duodenitis.

The other endoscopic findings were encountered occasionally and similarly in children with CMA and RAP. Endoscopic esophagitis was diagnosed in five (6%) and gastric erosions, encountered mainly on the mucosa of the antrum or pylorus, in 17 (21%) of the 80 subjects examined with gastroduodenoscopy. Altogether 14 (64%) subjects with untreated CMA, 12 (86%) with treated CMA and 21 (48%) with RAP exhibited at least one positive endoscopic finding.

Levels of cow's milk antibodies

As demonstrated in Table 2, the subjects with untreated CMA showed significantly higher levels

of IgG class antibodies to β LG, BSA and α CAS. The IgA class antibodies to whole milk and β LG were also found to be significantly higher. Detectable titers of IgA antibodies to BSA were observed in 31% of the study subjects (with untreated and treated CMA) and 28% of the controls (RAP and non-RAP).

In general, the levels of both IgA and IgG antibodies to specific milk proteins were lower in the subjects with treated CMA than in the untreated cases. A comparison of their titers with the controls showed the difference to be quite similar to that between the untreated cases and controls.

Interestingly, the patients examined for RAP showed much the similar pattern of both IgA and IgG class milk antibodies than the CMA children. There was a highly significant difference in both IgA and IgG class antibodies to whole milk between the RAP patients and controls.

Lymphonodular hyperplasia and cow's milk antibodies

The patients with untreated CMA or RAP and with LNH of the duodenal bulb as the main endoscopic finding showed a significant rise in the IgG class antibodies to β LG ($p = 0.005$), BSA ($p = 0.014$), and α CAS ($p = 0.0001$), and the IgA class antibodies to β LG ($p = 0.0001$) as compared with the non-atopic controls. The IgG class antibodies to α CAS showed the biggest difference (Fig. 2).

Discussion

The present study suggests that an intensive humoral immune response to cow's milk proteins and their fractions may associate with gastrointestinal symptoms even in children over the age of 3 years. The endoscopic findings of these patients included local and patchy hyperplasia of lymphoid tissue, and the immunological evidence showed the untreated CMA patients to have significantly higher levels of IgA and IgG class antibodies to whole cow's milk and its specific fractions than age-matched controls. The latter result was also mostly true of the CMA subjects on an elimination diet at the time of the study, though IgA class antibodies showed marked variation. The subjects with LNH as an endoscopic finding had the highest levels of both of these antibodies.

Another interesting new finding was that the patients with RAP but no symptoms of immediate CMA included six subjects with LNH of the duodenal bulb. These patients as a group also showed similar high levels of IgG and IgA class

antibodies to whole cow's milk and its specific fractions as the subjects with CMA and had significantly higher values than the controls. CMA was sought for only in children with several intestinal symptoms, while those presenting solely with abdominal pains were not subjected to an elimination challenge trial with cow's milk. Still, the finding of similar patterns of IgG and IgA class antibodies to cow's milk and frequent lymphonodular hyperplasia suggest that in some of them the symptoms may be due to cow's milk allergy.

As reported earlier, LNH of the duodenal bulb was the main endoscopic finding in half of the subjects with untreated CMA. It was also seen in treated CMA patients as well as in some of the RAP patients. This and the other endoscopic and histological biopsy findings serve as evidence of the presence of local immunological activity on the mucosa of the small intestine. Interestingly, Wakefield and his colleagues reported LNH of the terminal ileum as a common endoscopic finding in children with developmental disorders (10). The same group later showed in the same patients enhanced local immune response with a high density of intraepithelial $\gamma\delta^+$ TCR-bearing T cells (11). We reported the same from the duodenum (12). Our conjecture is that LNH is a common and patchy finding anywhere on the GI tract. It seems to reflect enhanced immune responses against luminal antigens, but as found also in treated cases of CMA the anatomic LNH seems to sustain even in states of immunological balance. Finally, so far the true prevalence and significance of LNH in healthy and disease states remains unknown and further investigations are necessary to clarify this curious phenomena.

However, the elevated concentrations of IgG and IgA class antibodies could not be uniquely considered to associate with LNH or any other endoscopic or histologic findings, as there was wide inter-individual variation in all of these values. An obvious explanation for this wide variation is that the mucosal alterations in subjects with gastrointestinal food hypersensitivity are patchy or segmental and may be found at any location between the stomach and the anus and to any extent. As the immunological responses as well as the mucosal protection against influxing antigens vary, the clinical characteristics may also vary.

Antibodies to cow's milk are produced by all individuals who consume milk. In healthy children, the IgA and IgG titers are associated with the time of introduction of cows milk into the diet; they are higher when cow's milk is

started early (13,14). The titers reach their highest values a few months after the dietary introduction of cow's milk. In healthy children, both humoral and cellular immune responses to cow's milk and its protein fractions get weaker after the age of 1 year, and we speculated that children begin to tolerate these proteins as they continue to ingest them (15). The oral tolerance thus developed may be diminished, at least in experimental animals, by gamma/delta T cells from a non-tolerant animal (16). These cells have been found in elevated quantities in the epithelium of patients with CMA and intestinal symptoms (12,17). Abrogation of tolerance leads to enhanced immune responses to the antigens and may be associated with intestinal pathology and even with autoimmune diseases (18). We speculate that the significant elevation of IgG and IgA antibodies to several fractions may present a consequence of the loss of oral tolerance. The associated symptoms might then be due to an abnormal immune reaction to cow's milk. Another possibility is that children with a delayed type reaction to cow's milk show increased permeability of the intestine and, hence, increased antibodies, as seen in such states as viral gastroenteritis and IgA deficiency (19,20). We recently demonstrated that the levels of cow's milk antibodies also correlated with the amount of cow's milk ingested by the individual (21). The tendency of children with untreated CMA to restrict their intake of cow's milk, would therefore lower their antibody titers. Even patients with diagnosed CMA mostly had elevated levels of CM antibodies. The dietary effect in both groups thus alleviated the observed difference. Whether these antibodies really have a pathogenetic role in the hypersensitivity cascades remains to be elucidated. Jenmalm with co-workers found a significant association between a high titer of IgG antibody levels to β LG and the manifestation of atopic symptoms at 6 and 18 months of age, suggesting that intermediate type hypersensitivity may have a pathogenetic role in atopic manifestations (7).

In concordance with other studies, the histological alterations in the mucosal samples of children with CMA after 3 years of age were scarce, with none showing either signs of slight villous atrophy and crypt hyperplasia or abundance of eosinophilic cells, both of which are typical findings in CMA in infants (22,23). A slight increment of eosinophilic cells was seen in a certain number of CMA subjects, who still had a normal villous pattern. The abundance of lymphoid nodules was a typical feature of these patients. As described more in detail very recently

CMA after infancy seems to switch on from immediate atopic allergy into local, gastrointestinal and immunologically into more delayed type (24). The rise of IgG and IgA class antibodies seem to be typical to the gastrointestinal CMA whereas those with immediate symptoms may have even low values. This variety of the background immune mechanisms most probably explains also some of the variety in the antibody levels.

An atopic, IgE-mediated reaction to food proteins, particularly to cow's milk proteins, is typical of food allergy during infancy. Typically, this response is elicited by small amounts of cow's milk given early and infrequently to an infant with a hereditary predisposition to atopy (25,26). IgE-mediated reactions to food proteins become more rare with increasing age. IgE-mediated CMA seldom manifests after the age of 1 year. The early development of this type of CMA is explained by the predominance of T-helper-2 skewed immune responses in the new born infant, which are changed towards T-helper-1 dependence during the early months of life (27). The rapid development of the intestinal immune system and the loss of permeability of proteins may also change the immune-mediated response to food proteins (28,29). Even in infancy, CMA with slowly developing intestinal symptoms is rarely mediated by IgE antibodies (12,23). None of our patients showed IgE antibodies to cow's milk. Like in infants with delayed intestinal symptoms, other immune mechanisms are likely to operate in them. This assumption is consistent with Yabuhara et al. who showed that most of the children with IgE-specific food antibodies in infancy down-regulate this reactivity by the age of 5 years (30). CMA may persist even after that, but it is mediated by other immunological mechanisms and associated with patchy intestinal inflammation. Very recently, Pelto et al. showed that CMA may be found even in young adults, all of whom have a pure gastrointestinal type of the disease (31). In this study, we also found evidence that, in a certain proportion of subjects with RAP as the main symptom, gastrointestinal milk allergy may actually be the main operative pathomechanism. They may actually be 'very slow responders'.

In conclusion, we found endoscopic and immunological evidence that CMA may be one state behind LNH of the duodenum and RAP in children. Further, epidemiological and immunohistochemical studies are, however, necessary to understand more in depth the underlying spectrum of reasons for LNH, and the significance of

immune responses produced by food allergens for the gastrointestinal complaints.

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