

Increased IFN- γ secretion from duodenal biopsy samples in delayed-type cow's milk allergy

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A delayed and local gastrointestinal hypersensitivity to cow's milk (CM) protein is difficult to diagnose and there are limited data on this disorder. The aim of this study was to investigate local intestinal cytokine secretion in the upper small intestine in children with delayed-type cow's milk allergy (CMA). Duodenal biopsy samples from 31 children with delayed CMA, 14 with celiac disease (CD), and 14 healthy controls were studied for the spontaneous release of IFN- γ , TNF- α , IL-2, IL-4, IL-5, and IL-10, measured by cytometric bead array, and of TGF- β and IL-6 measured by ELISA. The children with delayed CMA secreted more IFN- γ than the controls ($p = 0.006$) and the children with CD ($p = 0.006$). The children with CD secreted more IL-6 compared to the controls ($p = 0.008$) and the children with delayed CMA ($p = 0.002$). The children with delayed CMA who had continuously been exposed to CM secreted less TGF- β than the children with delayed CMA who avoided CM ($p = 0.050$), and showed a tendency towards lower secretion compared to the controls ($p = 0.078$). Secretions of TNF- α , IL-2, IL-4, IL-5, and IL-10 were low in general; however, the children with delayed CMA who did not avoid CM secreted more IL-4 and IL-10 than the controls ($p = 0.016, 0.059$). In conclusion, the children with delayed CMA showed up-regulation of IFN- γ . Interestingly, TGF- β secretion was up-regulated in those children with delayed CMA who avoided CM suggesting recovery of regulation mechanisms.

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Food antigen-related hypersensitivities occur in individuals when the development of normal oral tolerance fails or well-balanced tolerance breaks down. Intestinal diseases are often associated with an increased permeability to macromolecular food antigens, which after penetration to the intestinal lumen can stimulate the underlying immune system. The release of cytokines and inflammatory mediators further enhances the degradation of the epithelial barrier, leading to a vicious circle of inflammation (1). Gamma interferon (IFN- γ), the best known example of inflammatory cytokines, has been shown to disrupt tight junctions and to increase the paracellular permeability of the intestinal epithelium (2, 3). In celiac disease (CD) IFN- γ , as well as

other Th₁-associated cytokines but also Th₂-type cytokines (e.g., IL-4) and macrophage-derived cytokines (e.g., TNF- α), have been shown to be up-regulated in association with a massive increment of intraepithelial CD3⁺, $\alpha\beta$ ⁺, and $\gamma\delta$ ⁺ T cells (4–8). The activation of the immune cascades in delayed-type gastrointestinal (GI) cow's milk allergy (CMA) are much weaker and poorly characterized, but there also seems to be an accumulation of lymphoid cells in the form of nodules, a mild increment of intraepithelial T cells and an up-regulation of both Th₂- and Th₁-type cytokines (8–12).

In this study we investigated the local small intestinal immune responses in children with delayed-type GI CMA, and compared them with

those in children with CD and healthy control children, by determining the spontaneous secretion of IFN- γ , TGF- β , TNF- α , IL-2, IL-4, IL-5, IL-6, and IL-10 from biopsy specimens taken from the duodenal bulb.

Subjects and methods

Subjects

A total of 59 children were examined (median age 10 yr, range 1–18 yr, 28 males, Table 1). All the children had been referred to Oulu University Hospital, Finland, for a pediatric gastroenterological consultation because of recurrent GI complaints. The children underwent a gastro-duodenoscopy performed by the same pediatric gastroenterologist (JK). Thirty-one children were diagnosed to have delayed-type CMA, and 14 to have CD, while 14 children with negative cow's milk (CM) and cereal challenges and normal endoscopic findings served as controls. At the beginning of the study, i.e., when the children were examined for the first time, 17 of the children with delayed CMA consumed CM normally and 14 avoided it. Endoscopies were performed before any food challenges.

Diagnosis of delayed CMA and CD

The diagnosis of hypersensitivity towards CM protein was based on the disappearance of symptoms during the CM elimination diet (4 wk) and their reappearance in an open challenge test, which was to continue for another 4 wk with low lactose or lactose free milk and dairy products if symptoms did not appear earlier. The gold standard, blinded challenge, was considered too time-consuming and laborious because the symptoms of the children were

consistently delayed. Either the children or their parents kept written symptom diaries. Abdominal pain, diarrhea or loose mucous stools were each considered a positive result. Exacerbation of dermatitis was also marked into the diary.

Celiac disease was diagnosed according to the acknowledged criteria (13). All the patients had a positive titre of IgA class antibodies against transglutaminase, at least subtotal or total villous atrophy with crypt hyperplasia, and mononuclear infiltration in the lamina propria.

Endoscopic examinations and samples

Mucosal biopsy samples from the proximal duodenum were taken during an endoscopic examination performed under general anesthesia, as is the normal procedure in the hospital, using an Olympus GIF-XQ 140 gastroscope (Olympus, Tokyo, Japan). Biopsy samples were drawn for routine histology from at least four separate areas. Additional specimens were obtained from the sites where local disease alterations were seen.

The assessment of lymphoid nodularity on the duodenal mucosa was based on endoscopic evaluation after filling the area to be inspected with air. Only a cluster of lymphoid nodules ($n > 10$ nodules) was considered significant.

Immunohistochemical stainings to calculate the density of intraepithelial T cells (CD3+, $\alpha\beta$ +, and $\gamma\delta$ +), were performed as described in detail previously (11). Unfortunately, immunohistochemical data were not available from each patient.

Cytokine release from duodenal biopsy samples

Immediately after endoscopy each biopsy specimen was incubated in 0.5 ml of RPMI medium in a CO₂ incubator for 5 h at a temperature of 37 °C. The supernatant was collected by centrifugation, frozen with liquid nitrogen, and kept at –70 °C until analyzed.

TGF- β was measured with a Quantikine® human TGF- β 1 ELISA kit according to the instructions of the manufacturer (R&D Systems Inc., Minneapolis, MN, USA). The detection line of the kit was 7 pg/ml. IL-6 was measured with a PeliKine Compact™ human IL-6 ELISA kit, the detection line of which was 0.6 pg/ml, according to the instructions of the manufacturer (Central Laboratory of the Netherlands Red Cross, Amsterdam, Netherlands).

The BD Cytometric Bead Array Th₁/Th₂ kit (BD Biosciences, San Diego, CA, USA), with a detection line of 2.5 pg/ml, was used for the measurement of IFN- γ , TNF- α , IL-2, IL-4, IL-5,

Table 1. Baseline characteristics of the study subjects

	Delayed CMA 1	Delayed CMA 2	CD	Controls	Total
Characteristics					
N	17	14	14	14	59
Male/female	8/9	9/5	8/6	3/11	28/31
Age, years (range)	10 (4–15)	8 (2–18)	11 (1–14)	12 (1–15)	10 (1–18)
Main GI symptoms					
RAP	9	4	5	10	
Diarrhea	6	6	4	1	
Hematochezia	2	1	1	–	
Retarded growth	–	2	1	1	
Other GI symptom	–	1	3	2	

GI, gastrointestinal; RAP, recurrent abdominal pain; CD, celiac disease. CMA 1 refers to children with delayed CMA who consumed CM before endoscopy and CMA 2 to children who avoided CM.

and IL-10 in the supernatants according to the manufacturer's instructions. Samples were processed in duplicate. The samples from some children were not large enough for all the measurements.

Statistics

The primary variables were the concentration of cytokines (pg/ml) and the densities of the intraepithelial T cells. Because of skewed distribution of cytokines, results are presented as medians, and logarithmic (ln) transformation was used prior to statistical analysis. The conventional one-way anova and a *post hoc* test were used to estimate the significance of differences between the groups. The Pearson correlation coefficient was used to analyze the association between untransformed cytokine data. A $p < 0.05$ was considered significant.

The study protocol was approved by, the Ethics Committee for Clinical Science of Oulu University Hospital. Informed consent was obtained from all the parents of the study children, and also from the children if appropriate.

Results

Endoscopic findings and histological findings

Lymphonodular hyperplasia was a characteristic endoscopic finding in 17/31 (55%) children with delayed CMA, and no difference was found between children who did or did not avoid CM (8/14 vs. 9/17). Lymphonodular hyperplasia was found in 1/14 (7%) children with CD but in none of the controls (all groups $\chi^2 = 18.5$, $p < 0.001$). None of the children with delayed CMA or the controls showed duodenal villous atrophy, crypt hyperplasia or mononuclear infiltration, all of which were seen in 11 out of 14 children with CD.

Intraepithelial T cells

The densities of intraepithelial T cells were up-regulated in the food hypersensitivity groups (delayed CMA and CD, Table 2). The children with CD had by far the highest densities of CD3+, $\alpha\beta$ + and $\gamma\delta$ + T cells and an elevated $\gamma\delta$ +/CD3+ ratio, compared with the other groups ($p < 0.001$ for all). Even though the difference between the children with delayed CMA on different diets was not statistically significant, up-regulation of the T cells was more prominent in the children exposed to CM than in those who avoided CM: in the former the

Table 2. Median densities of intraepithelial T cells in the duodenal biopsy specimens (range)

	CD3+	$\alpha\beta$ +	$\gamma\delta$ +	$\gamma\delta$ /CD3+
Delayed CMA 1 (n = 13)	23.0 (8–35)*	22.0 (8–26)**	1.4 (0–8)	7.8 (0–30)
Delayed CMA 2 (n = 10)	20.5 (10–35)	17.5 (8–25)*	1.8 (0–5)	9.1 (0–23)
CD (n = 12)	49.0 (2–76)***	33.0 (5–54)***	8.2 (0–29)***	27.3 (0–55)***
Controls (n = 8)	13.0 (5–16)	9.5 (0–15)	0.8 (0–3)	6.6 (0–23)

p-Values compared to controls are presented as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CMA 1 refers to children with delayed CMA who consumed CM before endoscopy and CMA 2 to children who avoided CM.

densities of CD3+ ($p = 0.046$) and $\alpha\beta$ + cells ($p = 0.009$), and in the latter the densities of $\alpha\beta$ + cells ($p = 0.047$) were higher compared to the controls.

Cytokine secretion pattern in delayed CMA and CD

The children with delayed CMA secreted more IFN- γ than the controls (median: 33.5 vs. 10.0 pg/ml; $p = 0.004$), and more still than the children with CD (33.5 vs. 14.4 pg/ml, $p = 0.005$). The secretions of IL-4, IL-5, IL-10, and TNF- α were low in general. However, even though the concentrations were low (the detection limit 2.5 pg/ml), the children with delayed CMA who consumed CM secreted more IL-4 (5.5 vs. 2.7 pg/ml, $p = 0.016$) and slightly more IL-10 (4.6 vs. 2.8 pg/ml, $p = 0.059$) than the controls. The children with delayed CMA who consumed CM also showed lower secretion of TGF- β than the children with delayed CMA who avoided CM (399.5 vs. 612.3 pg/ml, $p = 0.050$), and a tendency towards lower secretion compared to the controls (399.5 vs. 441.1 pg/ml, $p = 0.078$, Fig. 1).

The children with CD released more IL-6 compared to the controls (23.8 vs. 3.1 pg/ml, $p = 0.008$) and the children delayed CMA (23.8 vs. 3.5 pg/ml, $p = 0.002$).

The release of IFN- γ showed a positive correlation with IL-10 in all the groups (Fig. 2).

Discussion

This study showed a distinctive pattern of intestinal immune activation in children with delayed-type CMA with GI symptoms. The cytokine environment of the intestine is usually studied by immunohistochemistry or *in situ* hybridization, which reveal the number of

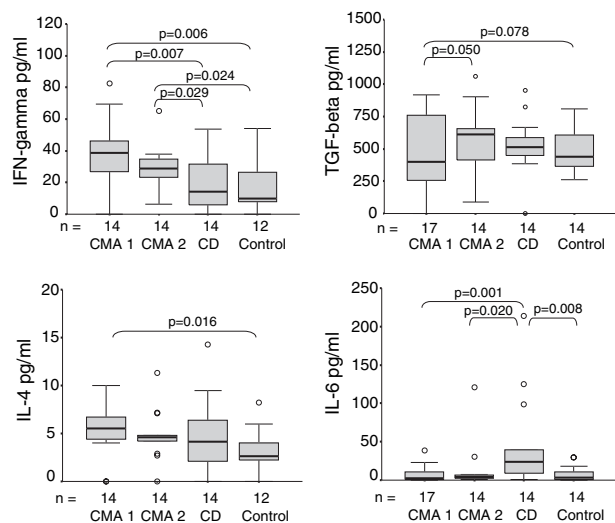


Fig. 1. Release of IFN- γ , TGF- β , IL-6, and IL-10 from duodenal biopsies, in children with delayed-type CMA, or CD, and control children, presented as box-plot figures. CMA 1 refers to children with delayed CMA who consumed CM before endoscopy and CMA 2 to children who avoided CM. Boxes indicate the lower and upper quartiles with medians, vertical lines represent the minimum and maximum values, and outliers (>1.5 box length from the end of the box) are marked as circle.

cytokine-specific cells in the tissue. It is possible that the amount of these cells in tissue does not directly correlate with the amount of cytokine secreted, since the method does not reflect the activity of the cytokine-secreting cells. The amount of cytokine or the balance between the cytokines in the tissue is thus difficult to estimate by these methods. Our approach was to measure the release of cytokines from biopsy samples in order to get a picture of the network of cytokines secreted in the intestinal environment. Since we did not stimulate the biopsies with any form of mitogen or antigen during the incubation, the result emphasizes the biological significance of these intermediates.

The studies of intestinal cytokine profiles in delayed-type GI CMA are not consistent. Our finding of increased IFN- γ release in the duodenum in the children with delayed CMA is in accordance with a study by Veres et al. (8), in which children with untreated delayed-type food allergy to CM and/or cereals had elevated densities of IFN- γ cells and a high expression of IFN- γ mRNA in the duodenum, detected by immunohistochemistry and *in situ* hybridization. These findings suggest that the activation of Th₁ immune response is also associated with GI hypersensitivity. Hauer et al. (10) showed increased frequency of IFN- γ but also of IL-4 secreting cells, and decreased frequency of IL-10 secreting cells, detected by ELISPOT, in

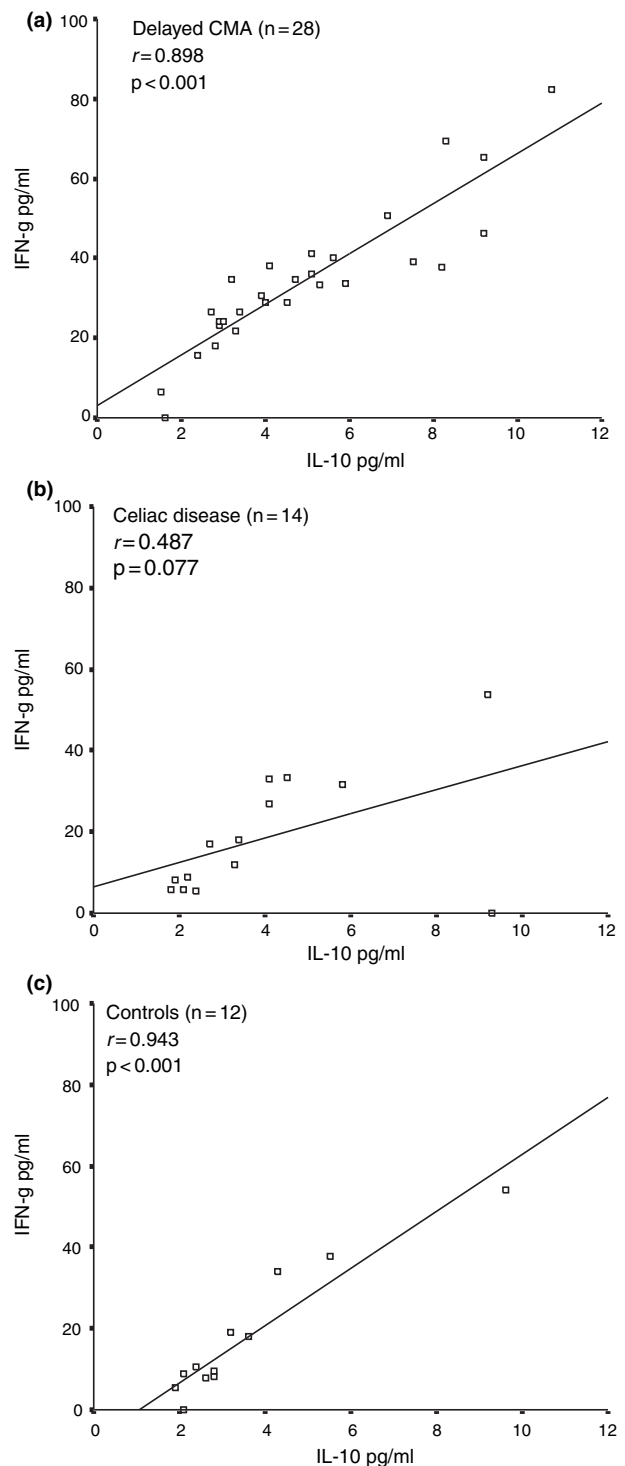


Fig. 2. Correlation of IFN- γ and IL-10 in samples derived from the duodenum in (a) children with delayed-type CMA, (b) children with CD, and (c) control children. The line is a linear regression line.

duodenal samples of young children with CM sensitive enteropathy, a local hypersensitivity, suggesting that local intestinal cytokine activation characterized by both Th₁ and Th₂ cells may

be associated with GI CMA. On the other hand, Lin et al. (14) found reduced numbers of IFN- γ + cells and increased numbers of IL-4+ cells, detected by immunostaining, in duodenal biopsies of symptomatic food allergic adults with negative serum IgE, suggesting localized IgE-mediated response in the GI tract during the symptomatic period of these patients. When we studied cytokine mRNA expression by RT-PCR in delayed-type CMA, we found increased expression of IL-6 and chemokine receptor 4 in duodenal biopsy samples, but not of IFN- γ (Paajanen et al., unpublished data). In the present study the release of IL-4 seemed to be slightly up-regulated in the children with delayed CMA who consumed CM. However, as the release of another Th₂ cytokine IL-5 was very low in all the study groups, we are not able fully to determine the extent of local Th₂ activation in delayed CMA, because of problems with the sensitivity of the assay. Nevertheless, local intestinal immune activation in delayed CMA was dominated by, though not restricted to, the Th₁ response.

It should be noted that the number of studies on delayed-type GI CMA is limited, and clinically heterogeneous patients were included in these studies, which may explain the discrepancies between the results. These discrepancies may also be partly explained by methodological differences, since the number of cytokine-stained cells, mRNA measurements and cytokine secretion assays often give controversial results. The protein secretion assays probably give the most relevant results in the biological significance sense. Thus, our results on the release of cytokines may reveal new insights.

In the present study IL-10 release also tended to be higher in the children with delayed CMA who were normally exposed to dietary CM compared to the controls. The role of IL-10 in the inflammatory process is dual, since it has both anti-inflammatory and pro-inflammatory potential. The role of IL-10 in the intestinal homeostasis is evidenced by studies on IL-10 deficient mice, which develop chronic inflammation in the intestine with no obvious inflammatory lesions elsewhere (15). However, the anti-inflammatory role of IL-10 as a primary factor in the intestine has recently been questioned (16). A report by Fuss et al. (16) argues that the protective effect of IL-10 is merely an indirect consequence of its effect on TGF- β secretion. Increased numbers of IL-10 expressing CD3+, CD8+, T cells in the ileal lamina propria lymphocytes have been reported in patients with spondyloarthropathy (17). In our

study, the release of IL-10 correlated with the levels of IFN- γ in all the study groups, which suggests that IL-10 reflects inflammation in human mucosa, as earlier reported in studies on rheumatoid arthritis and spondyloarthropathy (17, 18).

When we compared the children with delayed CMA who avoided or were exposed to CM before endoscopy, the latter showed a down-regulation of TGF- β , which supports earlier findings of down-regulation of TGF- β in food-hypersensitive children (19, 20). TGF- β is an inhibitory cytokine recognized as a key regulator of immunological homeostasis and inflammatory responses, and is associated with the development of oral tolerance (21). It inhibits T-cell proliferation and is also a product of mucosal regulatory T-cell population (22).

We did not observe elevated IFN- γ release in the children with CD, even though IFN- γ up-regulation is a constant finding in this disease, and has been reported even in patients with mild or potential cases (4–7). To our surprise the children with delayed CMA showed a higher release of IFN- γ than was seen in the children with CD. Our results may have been affected by *in vivo* stimulation of the intestinal immune cells, i.e., by exposure to an antigenic trigger (CM proteins or wheat gluten) preceding the biopsy. Our finding of increased IL-6 secretion in CD is in accordance with the literature: increased expression of IL-6 has been reported in cases of untreated CD, in some studies normalizing during a gluten elimination diet (4, 23, 24). IL-6 is secreted by several cells including monocytes, and contributes to mucosal IgA production (25). IL-6 seems to be a cytokine associating in particular with the accumulation of lymphoid cells on the mucosa of the gut.

In conclusion, delayed-type CMA was characterized by, a local intestinal cytokine network consisting of high IFN- γ secretion. Moderate IL-4 and IL-10 activation and decreased TGF- β activation was seen in the children with delayed CMA who were exposed to CM. In the children with delayed CMA who avoided CM in their diet, recovery of regulatory cytokine TGF- β was seen.

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