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Changes in biomarkers during a six-month oral immunotherapy intervention for cow`s milk allergy

Susanna Salmivesi, MD,¹ Marita Paassilta, MD, PhD,¹ Heini Huhtala, MSc,²

Riina Nieminen, PhD,³ Eeva Moilanen, MD, PhD,³ and Matti Korppi, MD, PhD,^{4,5}

1 Allergy Center, Tampere University Hospital, Tampere, Finland

2 School of Health Sciences, University of Tampere, Tampere, Finland

3 The Immunopharmacology Research Group, University of Tampere

4 School of Medicine and Tampere University Hospital, Tampere,
Finland

5 Tampere Center for Child Health Research, Tampere University and
University Hospital, Tampere, Finland

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Correspondence: Susanna Salmivesi, Allergy Center, Tampere University
Hospital, Teiskontie 35, 33521 Tampere, Finland

E-mail: susanna.salmivesi@pshp.fi

Abstract

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Aim: Oral immunotherapy (OIT) is a promising but still experimental method to treat children with cow's milk (CM) allergy (CMA). We evaluated changes in allergic, immunological and inflammatory parameters, which happened during the six-month OIT for CMA.

Methods: We treated 28 school-aged children with CMA using OIT with a double-blind placebo-controlled design. After the controlled study finished, the placebo group was treated with the same but open-label OIT protocol. Sixteen immune variables were tested before and after the six-month OIT.

Results: Before OIT, the median serum CM-specific immunoglobulin (Ig) E was 18.0kIU/L in the intervention group and 9.4kIU/L in the placebo group ($p=0.46$). At six months, interleukin (IL)-6 and IL-10 were significantly higher in the intervention group. When the changes during the blinded and open OIT were analyzed together for both groups, blood eosinophils and serum total IgE decreased and milk-specific IgG and IgG4, serum IL-4 and IL-6, and serum leptin and resistin increased significantly.

Conclusion: Preliminary evidence was found that markers of allergy like blood eosinophils and serum IgE decreased and milk-specific IgG and IgG4 increased during OIT. Adipokines leptin and resistin, which functionally are cytokines linked to Th1-type response, increased during OIT.

Keywords: Adipokines, Cow's milk allergy, Cytokines, Eosinophils, Immunoglobulin E, Oral immunotherapy

Keynotes:

- Oral immunotherapy (OIT) is an experimental treatment for food allergy. The mechanism of OIT is not known.
- Blood eosinophils and serum IgE decreased and milk-specific IgG and IgG4 increased during OIT. Th2-type IL-4, regulatory IL-10 and pro-inflammatory IL-6 cytokines, and inflammatory adipocytokines leptin and resistin increased.
- We found that OIT induced some beneficial immunological changes. A novel finding was that leptin and resistin, linked to Th1-type response, increased during OIT.

Cow's milk (CM) allergy (CMA) is the most common childhood food allergy. High serum concentration of CM-specific immunoglobulin (Ig) E, or the presence of IgE to distinct epitopes of CM proteins, predicts the persistence of CMA (1,2). About 75% of children with CMA can tolerate heated forms of CM, and these patients are more likely to become tolerant over time (3). Consuming heated milk, oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) are promising therapeutic approaches. Several studies have confirmed that OIT can also be used for children with severe and persistent food allergies (4-8). Mild and self-limiting adverse reactions are frequent during OIT, but severe and life-threatening reactions may also occur (5,9).

The mechanisms leading to desensitization or even tolerance to food allergies are mainly unknown, but they may be similar to those seen in standard subcutaneous immunotherapy (SCIT). SCIT is an established treatment for pollen allergies, which releases IL-10 and transforming growth factor (TGF) - β from the T cells. In children

with peanut allergy, OIT has been shown to lead to weakened responses in skin prick tests (SPT), less antigen-specific basophil activation and decreased allergen-specific IgE concentrations in serum, and, correspondingly, to increased allergen-specific IgG1 and IgG4 and higher regulatory T-cell (Treg) counts and higher Treg-associated cytokine levels (10,11). In egg allergy, OIT has been shown to lead to increased egg-specific IgG4 in serum and weakened responses to SPTs, but no changes have been observed in egg-specific IgE (12). One study showed that when CMA was treated with a 16-week OIT programme, the SPT responses to CM and serum CM-specific and casein-specific IgE decreased when they were compared to the initial assessments (13). In an 80-week follow-up after milk OIT, another study showed that serum CM-specific IgE decreased significantly, but this was not seen in milk SLIT (14). Meanwhile, another study showed that short-term changes in milk-specific IgE were not seen, but milk-specific IgG1 and IgG4 increased (15).

T-helper (Th) lymphocytes are the major source of cytokines, and can be further divided into a number of subtypes, such as Th1 and Th2 (16). IL-12 is considered to be a key player in the development and maintenance of Th1 cells. Th2-type cytokines include IL-4 and IL-5, which are associated with the promotion of IgE-mediated and eosinophilic responses. IL-6 is a multifunctional pro-inflammatory cytokine produced in systemic inflammation. IL-10 seems to be more an anti-inflammatory than a pro-inflammatory cytokine and it enhances eosinophil cell death. Eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) are secreted by activated eosinophils (17).

Adipokines, such as leptin, adiponectin, adipon and resistin, are mediators secreted by adipocytes and macrophages (18). Leptin, adipon and resistin have pro-inflammatory properties, whereas adiponectin mainly has anti-inflammatory properties. Adiponectin is a hormone that regulates lipid and glucose metabolism and leptin plays a key role in the control of Treg proliferation (19).

Recently, we used OIT for 28 cow`s milk allergic schoolchildren for six months in a placebo-controlled intervention (6). Sixteen (89%) of the 18 children in the active group completed successfully the protocol. Subsequently, ten children of the placebo group were treated with an identical but open-label protocol, and all of them completed the protocol.

Our hypothesis was that during OIT, atopy and eosinophil activity markers, and the Th2-type cytokines that regulate these markers, would decrease, and the markers of Th1-type immunology and the respective cytokines would increase.

The aim of this study was to evaluate the role of allergy and immunology markers in CMA in schoolchildren and the changes in markers during the six-month prospective, placebo-controlled OIT. The markers we monitored were: total IgE, eosinophils, ECP, EDN, CM-specific IgE, milk-specific IgG and IgG4, Th2-type cytokines IL-4 and IL-5, Th1-type cytokine IL-12, regulatory cytokine IL-10, pro-inflammatory cytokine IL-6 and the adipokines adipon, leptin, resistin and adiponectin.

SUBJECTS AND METHODS

Subjects

As previously described (6), 28 children between six and 14 years of age were recruited to this randomized, double-blind, placebo-controlled OIT study. All of them had been treated with a complete avoidance diet for CMA from early childhood in the Department of Pediatrics, Tampere University Hospital. There were 18 children in the intervention and 10 in the placebo group. The diagnosis of CMA was based on either a positive challenge test or an anaphylactic reaction after accidental exposure during the preceding six months. In all cases, IgE-mediated mechanism had been confirmed with either positive >3 mm responses in SPTs or with CM-specific IgE >5kU/l in serum (6). Two children in each group failed to complete the six-month study, leaving 16 in the active treatment group and eight in the placebo group.

Laboratory methods

Blood samples were obtained from all patients before the start of the study and at the end of the study. In addition, blood samples were obtained from the original placebo group after they completed the open-label OIT at 12 months, which was performed immediately after the blinded phase of the study. One of the dropouts in the active group did not participate in the six-month study visit and occasional blood samples were not analyzed for technical reasons (i.e. not enough serum). Total eosinophils were counted in the blood samples by an automated cell counter and expressed as cells $\times 10^9/l$. Serum total IgE was determined nephelometrically using a Siemens N Latex IgE mono assay (Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany). Serum CM-specific IgE was measured using the CAP-FEIA technique (Phadia,

Uppsala, Sweden) and expressed as milligrams of allergen per litre (mgA/l). The rest of the samples were stored at -70°C until they were analysed. Serum concentrations of IL-4, IL-5 IL-6, IL-10, IL-12p70, EDN, adiponectin, adipsin, leptin and resistin were determined by enzyme immunoassay (EIA) by using commercial reagents. IL-4, IL-5, IL-12p70, adiponectin, adipsin, leptin and resistin were measured by the DuoSet ELISA (R&D Systems Europe Ltd, Abindgon, Oxfordshire, U.K), IL-6 and IL-10 (PeliPair ELISA, Sanquin, Amsterdam, the Netherlands), EDN with the EDN ELISA kit (MBL International, Woburn, MA, USA), and ECP with the ECP ELISA kit (MBL International, Woburn, MA, USA). Serum allergen-specific IgG and IgG4 levels (Phadia AB, Uppsala, Sweden) were measured by the Immuno CAP 100 assay (Phadia AB, Uppsala, Sweden).

Statistics

Statistical analyses were performed with SPSS statistical software for Windows, version 20.0 (SPSS Inc, Chigago, IL, USA). The Mann-Whitney U-test was used to compare continuous variables between the two groups and the Wilcoxon test was used to compare continuous variables within the groups. The results for the two groups are given both separately and combined. The baseline was the same for both groups, but the outcome for the original placebo group was at 12 months, rather than six, when their phrase of the open OIT ended. The protocol was the same in the double-blinded and open OITs. We considered a two-tailed p value of less than 0.05 as statistically significant.

Ethics

Ethics approval for the study was obtained from the Ethics Committee of Tampere University and University Hospital. The children and their parents provided written informed consent. The study was registered at clinicaltrials.gov NCT01361347.

RESULTS

At the baseline, there were no significant differences between the active OIT group and the placebo control group in the immunity and inflammatory markers (Tables 1 and 2).

The only exception was the average milk-specific IgG4 level, which was higher in the intervention group. This may have been due to by chance because of large variation in that variable (Table 1).

At six months, serum IL-6 and IL-10 levels were significantly higher in the group that had been treated with OIT than in the control group (Table 2). There were no other significant differences in the immunity and inflammatory markers between the two groups (Tables 1 and 2).

When the markers in the active group were compared at baseline and at six months after the OIT intervention, there were no significant differences in any of the markers (Tables 1 and 2).

The control group was placed on the open-label OIT intervention after six months. This started immediately after the double-blind OIT study and used exactly the same protocol. Serum resistin was significantly higher at 12 months in this group than at the start of the six-month intervention (Table 3).

Finally, we combined the active and control groups and measured the values before either group took part in the OIT intervention, together with the six-month values for the first intervention group and 12-month values for the second intervention group, who acted as

the control placebo group for the first six months (Table 4). This showed that blood eosinophils and serum total IgE had decreased significantly after the intervention and allergen-specific IgG and IgG4, serum IL-4 and IL-6, and serum leptin and resistin had increased significantly.

DISCUSSION

We were able to confirm that serum total IgE, and blood eosinophils decreased significantly during OIT, when the data from the double-blind and open-label OITs were combined. Milk-specific IgG and IgG4 increased in line with this. Also in other studies, OIT has been associated with a rise in allergen-specific IgG4 (8,10,12). One indicator for successful milk-OIT seems to be an increase in serum IgG4 to milk components such as α -lactalbumin, β -lactoglobulin, casein and lactoferrin (20).

When immunotherapy with an allergen is effective, it changes the patient's allergen-specific response from an allergic (Th2) to a non-allergic (Th1) profile. This is promoted by Treg cells that release IL-10, which further induce IgG4, TGF- β and IgA production (16). As previously reported, the OIT intervention provided during the present study was clinically effective (6). OIT led to a desensitization, defined as the ability to tolerate more milk or milk products after than before treatment, in 89% of patients in the active group. In line with this, serum IL-10 was higher in the active group than in the control group at the end of the OIT study. However, the rise of IL-10 between the start and the end of OIT was not significant in either of the two groups or when the results for both groups were combined.

Infants with food-allergies have been reported to present with lower plasma IL-10 and

IL-6 than sensitized but tolerant nor non-sensitized infants (21). Defects in Treg cell activity, due to suppressed immune responses by regulatory cytokines such as IL-10 and TGF- β , may contribute to the development of food allergy (22). However, our patients were school-aged children, even though they had suffered from milk allergy since infancy. In a previous OIT study for peanut allergy, an upregulation of IL-10 was not seen, even though the children ranged from three to 14 years of age. This suggest that cytokine production may have been measured too late (8). In another peanut OIT study, IL-10 was measured immediately after OIT, like in our present study, and the increase in IL-10 was clearly seen (10). Treg cells secreting IL-10 and IL-6 are involved in the induction of desensitisation and tolerance in CMA (23), but we were only able to demonstrate a significant rise in IL-6.

To our knowledge, this is the first study that has documented that OIT affects serum adipokines. It has been reported that pre-seasonal SLIT courses significantly increased serum leptin in allergic rhinitis, but only in males (24). Our finding was that OIT resulted in significant increases in serum leptin and resistin levels and one explanation could be that leptin and resistin, as well as many cytokines, possess anti-allergic activity (18).

Leptin, at least, is known to influence cytokine production by switching the phenotype towards a Th1 response. Resistin has also been reported to act as an endogenous ligand of Toll-like receptor 4 (TLR4) that enhances classical macrophage activation and Th1-type response (25). Hence, increased resistin and leptin levels in our OIT intervention can be regarded as an effector and also a biomarker for the shift of Th2-type immune responses

towards Th1-type dominance. Future studies are warranted to confirm the role of leptin and resistin in food allergy and in monitoring the effect of OIT.

The results of this study should be interpreted with caution. The study population was small, which means that the study did not have enough power to find all the significant associations. The short follow-up period is another limitation and blood samples were only obtained at the start and end of OIT, not later as in many other OIT studies (5,8,10,12-14), where markers were monitored for one to 86 weeks after the active OIT. In one egg OIT study, children were assessed 4-6 weeks after they finished immunotherapy (12), and in a milk OIT *versus* milk SLIT study, patients were tested for sustained unresponsiveness at one and six weeks after therapy (14). Peanut OIT was also discontinued for two weeks before the final double-blind placebo-controlled food challenge to assess long-term tolerance (8). Monitoring hen`s egg and cow`s milk-specific IgE has been particularly useful for predicting prognoses after OIT (26). The rise especially in allergen-specific IgG1 correlated with good response in a previous Japanese egg OIT study (27).

CONCLUSION

This study provides preliminary evidence that markers of allergic inflammation, such as blood eosinophils and serum IgE decreased during milk OIT, while allergen-specific IgG and IgG4 and the pro-inflammatory cytokine IL-6 increased. Notable and novel findings were that leptin and resistin, which are two adipocytokines linked to Th1-type response, increased during OIT.

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Table 1. Immunity markers: comparisons between the intervention and placebo groups at the start of the study and at six months after the double-blind milk oral immunotherapy study ended

	Placebo group		Intervention group		p ¹	p ²	p ³
	Median (range)	n	Median (range)	n			
Baseline (0 months)							
Eos 10E9/l	0.86 (0.28–1.09)	9	0.58 (0.20–1.25)	17	0.287	0.031	0.302
IgE kU/l	731.00 (354.00–5040.00)	9	822.00 (228.00–10100.00)	18	1.000	0.078	0.804
CM IgE kU/l	9.40 (2.80–27.00)	8	18.00 (0.66–400.00)	18	0.461	0.094	0.793
ECP ug/l	30.10 (7.90–76.60)	9	19.50 (7.10–143.00)	17	0.560	0.109	0.229
EDN pg/ml	60.12 (38.41–186.00)	10	58.57 (21.83–272.20)	18	0.796	0.214	0.408
IgG mgA/l	75.70 (57.20–90.50)	10	75.05 (61.3–111.0)	16	0.816	0.086	0.638
IgG4 mgA/l	19.85 (16.10–24.00)	10	21.80 (13.70–112.40)	16	0.027	0.075	0.754
At 6 months							
Eos 10E9/l	0.43 (0.14–1.75)	8	0.43 (0.14–0.79)	16	0.742		
IgE kU/l	853.00 (308.00–3850.00)	8	831.50 (119.00–7280.00)	16	0.928		
CM IgE kU/l	6.65 (2.50–73.00)	8	11.00 (0.41–400.00)	16	0.528		
ECP ug/l	15.45 (6.90–50.40)	8	11.75 (6.40–31.90)	16	0.291		
EDN pg/ml	45.79 (25.49–115.36)	9	62.05 (24.20–120.02)	16	0.187		
IgG mgA/l	79.10 (62.50–141.00)	9	78.25 (65.50–123.0)	14	0.643		
IgG4 mgA/l	23.40 (18.50–216.00)	9	21.20 (15.00–238.00)	14	0.688		

p¹ value for intervention group vs placebo group 0 and 6 months

p² value for placebo group 0 vs 6 months

p³ value for intervention group 0 vs 6 months

Table 2. Inflammatory markers: comparisons between the intervention and placebo groups at the start of the study and at six months after the double-blind milk oral immunotherapy had ended

	Placebo group		Intervention group		p ¹	p ²	p ³
	Median (range)	n	Median (range)	n			
Baseline (0 months)							
IL-4 pg/ml	1.95 (1.95-1.95)	10	1.95 (1.95-7.30)	18	0.160	0.068	0.553
IL-5 pg/ml	2.05 (1.03-15.90)	10	1.90 (1.03-52.40)	18	0.555	0.889	0.955
IL-6 pg/ml	0.50 (0.30-3.40)	10	0.60 (0.30-9.20)	18	0.245	0.214	0.729
IL-10 pg/ml	1.85 (1.20-7.30)	10	2.65 (1.00-6.20)	18	0.191	0.401	0.134
IL-12p70 pg/ml	3.90 (3.90-4.80)	10	3.90 (3.90-3.90)	18	0.689	0.317	0.317
Adiponectin ug/ml	3.69 (1.76-7.57)	10	3.93 (2.09-6.84)	18	0.759	0.374	0.569
Adipsin ng/ml	833.56 (619.36-1109.94)	10	829.08 (597.35-1200.25)	18	0.869	0.859	0.877
Leptin ng/ml	3.64 (0.65-20.11)	10	3.83 (0.32-22.19)	18	1.000	0.859	0.215
Resistin ng/ml	3.94 (1.51-5.90)	10	2.98 (1.73-6.01)	18	0.382	0.260	0.501
At 6 months							
IL-4 pg/ml	1.95 (1.95-4.00)	9	1.95 (1.95-23.30)	16	0.329		
IL-5 pg/ml	1.30 (1.03-9.40)	9	2.00 (1.03-35.40)	16	0.251		
IL-6 pg/ml	0.50 (0.30-3.70)	9	0.95 (0.30-7.70)	16	0.032		
IL-10 pg/ml	1.50 (0.40-4.70)	9	2.25 (1.20-10.00)	16	0.023		
IL-12p70 pg/ml	3.90 (3.90-3.90)	9	3.90 (3.90-139.70)	16	0.637		
Adiponectin ug/ml	3.43 (1.34-5.31)	9	4.22 (2.05-7.43)	16	0.095		
Adipsin ng/ml	878.57 (703.31-1140.27)	9	879.21 (510.55-1139.32)	16	1.000		
Leptin ng/ml	5.14 (0.70-28.04)	9	4.32 (1.30-23.45)	16	0.846		
Resistin ng/ml	4.33 (1.30-7.56)	9	3.17 (1.61-5.70)	16	0.207		

p¹ value for intervention group vs placebo group 0 vs 6 months

p² value for placebo group 0 vs 6 months

p³ value for intervention group 0 vs 6 months

Table 3. Immunity and inflammatory markers in the initial control group: comparison of the results at the start of the study and after their subsequent open-label milk oral immunotherapy at 12 months

	Median (range)	n	Median (range)	n	p
Eos 10E9/l	0.86 (0.28-1.09)	9	0.47 (0.24-1.20)	10	0.044
IgE kU/l	731.00 (354.00-5040.00)	9	657.50 (340.00-3960.00)	10	0.173
CM IgE kU/l	9.4 (2.80-27.00)	8	9.55 (2.50-57.00)	10	0.176
ECP ug/l	30.10 (7.90-76.60)	9	36.85 (4.60-139.00)	10	0.214
EDN pg/ml	60.12 (38.41-186.00)	10	67.40 (37.00-159.70)	10	0.721
IgG mgA/l	75.70 (57.20-90.50)	10	96.25 (71.60-124.00)	8	0.012
IgG4 mgA/l	19.85 (16.10-24.00)	10	89.40 (19.00-114.40)	8	0.017
IL-4 pg/ml	1.95 (1.95-1.95)	10	1.95 (1.95-25.40)	10	0.068
IL-5 pg/ml	2.05 (1.03-15.90)	10	1.35 (1.03-6.80)	10	0.959
IL-6 pg/ml	0.50 (0.30-3.40)	10	1.45 (0.3-8.3)	10	0.014
IL-10 pg/ml	1.85 (1.20-7.30)	10	1.65 (0.7-5.8)	10	0.240
IL-12p70 pg/ml	3.90 (3.90-4.80)	10	3.9 (3.9-85.3)	10	0.317
Adiponectin ug/ml	3.69 (1.76-7.57)	10	2.75 (1.2-5.3)	10	0.059
Adipsin ng/ml	833.56 (619.36-1109.94)	10	898.80 (560.0-1290.60)	10	0.241
Leptin ng/ml	3.64 (0.65-20.11)	10	5.15 (1.00-19.70)	10	0.139
Resistin ng/ml	3.94 (1.51-5.90)	10	4.80 (2.10-8.60)	10	0.007

p value for placebo group 0 vs 12 months

Table 4. Immunity and inflammatory markers in the combined intervention and control groups. Comparison between pre-OIT values (at the start of the study) and post-OIT values (at six months in the intervention group and at 12 months in the former control group).

	Median (range)	n	Median (range)	n	p
Eos 10E9/l	0.60 (0.20-1.25)	26	0.41 (0.14-1.20)	26	0.003
IgE kU/l	778.00 (228.00-10100.00)	27	732.50 (119.00-7280.00)	26	0.048
CM IgE kU/l	12.00 (0.66-400.00)	26	10.40 (0.41-400.00)	26	0.088
ECP ug/l	20.55 (7.10-143.00)	26	16.30 (4.60-139.00)	26	0.853
EDN pg/ml	58.57 (21.83-272.20)	28	62.83 (24.20-159.70)	26	0.849
IgG mgA/l	75.05 (57.20-111.00)	26	83.15 (65.50-124.00)	22	0.039
IgG4 mgA/l	21.00 (13.70-112.40)	26	25.35 (15.00-238.00)	22	0.039
IL-4 pg/ml	1.95 (1.95-7.30)	28	1.95 (1.95-25.40)	26	0.011
IL-5 pg/ml	1.90 (1.03-52.40)	28	1.85 (1.03-35.40)	26	0.780
IL-6 pg/ml	0.60 (0.30-9.20)	28	1.00 (0.30-8.30)	26	0.008
IL-10 pg/ml	2.35 (1.00-7.30)	28	2.20 (0.70-10.00)	26	0.459
IL-12p70 pg/ml	3.90 (3.90-4.80)	28	3.90 (3.90-139.70)	26	0.109
Adiponectin ug/ml	3.87 (1.76-7.57)	28	4.05 (1.20-7.43)	26	0.970
Adipsin ng/ml	833.56 (597.35-1200.25)	28	879.21 (510.55-1290.60)	26	0.304
Leptin ng/ml	3.83 (0.32-22.19)	28	5.02 (1.00-23.45)	26	0.022
Resistin ng/ml	3.45 (1.51-6.01)	28	3.66 (1.61-8.60)	26	0.008

p value for placebo and intervention group combined 0 vs 6 or 12 months