

Measurements of Eosinophil Activation before and after Food Challenges in Adults with Food Hypersensitivity

J. van Odijk^{a,c} C.G.B. Peterson^{e,g} S. Ahlstedt^{g,h} U. Bengtsson^c M.P. Borres^{b,g}
L. Hulthén^a J. Magnusson^d T. Hansson^{f,i}

Departments of ^aClinical Nutrition and ^bPediatrics, Faculty of Medicine, ^cAsthma and Allergy Research Group, Department of Respiratory Medicine and Allergy, Sahlgrenska Academy at Göteborg University, and ^dDepartment of Rheumatology and Inflammation Research, Sahlgrenska University Hospital, Gothenburg; ^eDepartment of Medical Sciences, Clinical Chemistry, and ^fDepartment of Women's and Children's Health, Uppsala University, and ^gPharmacia Diagnostics AB, Uppsala, and ^hInstitute of Environmental Medicine, Center for Allergy Research, Karolinska Institute, and ⁱDepartment of Rheumatology, Karolinska Hospital, Stockholm, Sweden

Key Words

Eosinophil • Eosinophil protein X • Fecal samples • Food-induced gastrointestinal hypersensitivity • Leukotriene E₄ • Urinary samples

Abstract

Background: Objective assessment of inflammatory reactions in the gastrointestinal tract could be useful in the diagnosis of food hypersensitivity. The aim of the present study was to investigate the involvement of eosinophils and mast cells in the inflammatory response of patients with food hypersensitivity before and after food challenges. **Methods:** Eleven patients (4 with IgE-mediated allergy and 7 without) with food hypersensitivity and positive double-blind, placebo-controlled food challenge were subjected to food challenge in a single-blinded fashion. Four subjects with no known food hypersensitivity were recruited as controls. Placebo was given after a 1-week washout period followed by an active dose. Stool, urinary and serum samples were collected and symptoms were recorded in a diary. Fecal samples were analyzed for eosinophil protein X (F-EPX) and

tryptase; urinary samples for EPX (U-EPX) and leukotriene E₄ (U-LTE₄) and serum samples were analyzed for eotaxin and food-specific IgE antibodies. **Results:** Patients with IgE-mediated food allergy had increased levels of F-EPX compared to controls and tended to have lower serum levels of eotaxin compared to non-allergic patients and controls. U-LTE₄ was significantly higher in allergic patients compared to non-allergic patients after challenge. Moreover, F-EPX correlated to U-LTE₄ ($p = 0.011$). Reported symptoms, abdominal pain, distension, flatulence and nausea were similar in the allergic and non-allergic patients. **Conclusion:** The results strongly indicate that eosinophils are activated in the gastrointestinal tract of food-allergic patients but not in patients with non-allergic food hypersensitivity. Due to the inconsistent pattern of symptoms after placebo and active food challenge, it was not possible to relate the levels of inflammation markers to the recorded symptoms.

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J.v.O. and C.G.B.P. contributed equally to the study.

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Correspondence to: Dr. Christer Peterson
Department of Clinical Chemistry and Pharmacology
University Hospital
SE-751 85 Uppsala (Sweden)
Tel. +46 18 611 30 91, Fax +46 18 611 37 03, E-Mail christer.peterson@medsci.uu.se

Introduction

Adverse reactions to food are considered a major medical problem, with up to 35% of the population reporting reactions to food [1]. However, only 1–2.5% of the whole population have food hypersensitivity, as confirmed by double-blind, placebo-controlled food challenge (DBPCFC) [1, 2]. Diverse gastrointestinal symptoms occur in many patients with food hypersensitivity and reactions to food may cause unspecific symptoms such as nausea, vomiting, abdominal pain and diarrhea [3].

No single diagnostic test like skin prick test (SPT) or measurement of IgE antibodies in peripheral blood can offer an effective tool for the clinical diagnosis of food hypersensitivity. DBPCFC is considered the gold standard, although it does not discriminate between food allergy and non-immune-mediated food hypersensitivity [4, 5]. In addition, DBPCFC is considered time-consuming, costly and sometimes too risky to be used in daily practice [6]. During the last years some suggestions regarding standardization of challenge procedures have been made to facilitate and to increase reliability, but most of the proposals have been designed for immediate reactions only [7] though many patients have delayed and late reactions.

For adult patients with food hypersensitivity, with negative SPT and without serum IgE antibodies, hypersensitivity mechanisms are not well understood. Several studies have shown that hypersensitivity reactions to food antigens are associated with infiltration of inflammatory cells, e.g. eosinophil granulocytes and mast cells, that upon activation release mediators contributing to the pathogenesis of the disorders [8–10]. In food allergy and other inflammatory conditions affecting the gastrointestinal tract, eosinophils are predominant features of the intestinal inflammation [11].

Eosinophil protein X (EPX), one of the major proteins released by eosinophils [12], and tryptase, one of the major proteins released by mast cells during allergic reactions, can be accurately measured in feces [13, 14]. In a previous study including adult patients with food hypersensitivity, we observed an overall increase in the fecal levels of EPX (F-EPX) over time during periods of variable symptoms [15]. However, the measurements did not differentiate symptomatic from non-symptomatic periods.

In addition, EPX can be accurately assessed in the urine (U-EPX) [16] and shows a good correlation with eosinophil activation [17]. Increased levels of U-EPX have also been demonstrated in children with asthma

and atopic dermatitis and to correlate with disease activity [18, 19]. Furthermore, Gore et al. [20] showed that U-EPX reflects the presence of atopy and exposure to allergens, and U-EPX levels were associated with symptoms of allergic disease in children. However, data on the role of U-EPX in adults with food hypersensitivity are scarce.

Therefore, we further investigated the role of eosinophils and mast cells in food hypersensitivity by measuring inflammation markers in feces, urine and serum samples from adults with food hypersensitivity and positive DBPCFC in a single provocation model.

Patients and Methods

Patients

Eleven patients with food hypersensitivity verified with DBPCFC and gastrointestinal symptoms were collected from the Department of Respiratory Medicine and Allergology, Sahlgrenska University Hospital, Gothenburg, Sweden (fig. 1). The median age of the 10 females and the male was 44 years (range 28–70 years). Seven patients were considered having a non-IgE-mediated food hypersensitivity, and the remaining 4 had a documented IgE-mediated food allergy defined by positive levels of specific IgE antibodies and/or a positive SPT to common food allergens.

None of the study patients had positive serum levels of anti-tissue transglutaminase antibodies, and celiac disease was therefore excluded [21]. Six of the patients fulfilled the Rome II criteria of irritable bowel syndrome (IBS) [22], and 1 patient had an additional inflammatory bowel disease (proctitis).

All patients had previously been extensively examined and studied by means of diaries as well as elimination diets and open and blinded challenges. DBPCFC were performed with fresh food covered in drinks, depending on whether the expected symptoms were objective or subjective with two or six challenges on separate days [23]. The criterion for positive challenge includes appearance of expected symptoms at the active challenges and no symptoms at placebo. Some patients were challenged for several consecutive days since they were suspected to develop additional symptoms 3–5 days after exposure.

Previous DBPCFC revealed that 4 patients reacted to milk, 4 patients to wheat, 2 patients to meat, and 1 patient to benzoic acid (fig. 1). All allergic patients and 3/6 non-allergic patients had a history of symptoms like rhinitis, conjunctivitis and/or asthma during pollen exposure. The 2 patients with meat allergy also reported sensitivity to cats. Three out of 4 patients with milk hypersensitivity were SPT and serum IgE antibody negative for milk and all of them tolerated lactose.

Four healthy control subjects (median age 38 years, range 34–49 years) with no history of food hypersensitivity or celiac disease were also included. Informed consent was obtained from all the participants, and the Ethics Committee of the Medical Faculty of the University of Gothenburg approved the study protocol.

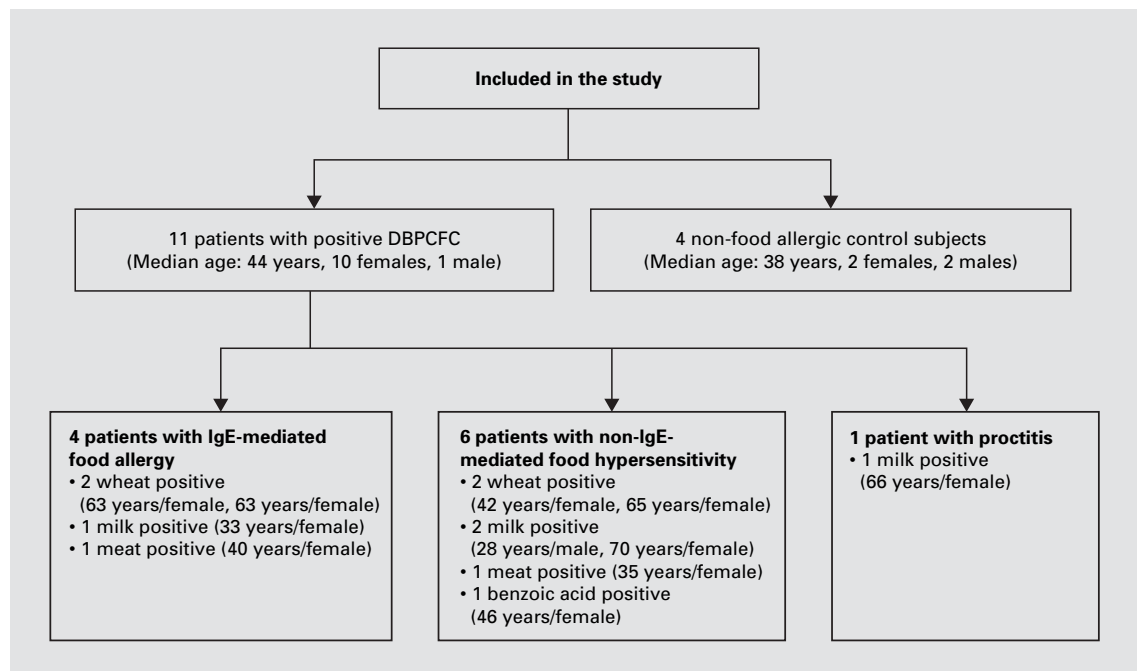


Fig. 1. Characteristics of the 11 patients and 4 controls included in the study.

Table 1. Study design

	Washout	Single-blind, placebo food challenge		Follow-up		Washout	Single-blind active food challenge		Follow-up		Washout
	day 1–7	before day 8	after day 8	day 9	day 10	day 11–14	before day 15	after day 15	day 16	day 17	day 18–21
Fecal sample		↑		↑	↑		↑		↑	↑	
Urine sample		↑	↑	↑	↑		↑	↑	↑	↑	
Serum sample		↑	↑				↑	↑			
Symptom diary	↑		↑	↑	↑	↑		↑	↑	↑	↑

Experimental Design

In the present study, all patients underwent renewed single-blind food challenges at the clinic. The patients started with a ‘washout’ week (7 days), while all foods that could cause symptoms were avoided. The following week the patients underwent a single-blind placebo food challenge and after another 3–5 days of elimination they returned for a single-blind active food challenge with the food they were DBPCFC positive for (table 1).

The food challenges were performed with foods mixed in drinks (except for benzoic acid, which was hidden in capsules). The drink was prepared at the clinic, and the amount of food was based on a normal serving portion per meal. The active and placebo doses were developed to resemble each other with regard to

taste, texture and color. All drinks were served cool in a covered cup and consumed through a straw.

Symptoms of abdominal pain, distension, flatulence and nausea were scored 1–3 by the subjects and registered in symptom diaries. The subjects were also requested to record motion frequency and stool consistency. During the whole study period, antihistamines, corticosteroids, leukotriene antagonists and sodium cromoglycate were avoided, but 4 patients took occasional medication and 1 patient had a cholesterol-lowering medicine during the challenge period. Samples of feces and urine were collected the morning before the first challenge and during the study (table 1).

Samples

Stool samples were collected in plastic tubes using a spatula in the patients' home and kept in the freezer until delivery to the clinic. Samples were then kept at -30°C , until analyzed. Processing of the stool samples was done according to Peterson et al. [13]. Briefly, approximately 0.1–1.5 g of thawed feces were weighed and diluted 10 times by adding 9 volumes (v/w) of an extraction buffer consisting of PBS, pH 7.4, supplemented with 10 mM EDTA, 0.2% N-cetyl-N,N,N-trimethylammonium bromide, 20% glycerol, 0.05% Tween-20, 1% BSA, a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Mo., USA) containing 4 mM 4-(2-aminoethyl)-benzensulfonyl fluoride, 0.26 mM bestatin, 28 μM E-28, 2 μM leupeptin and 0.6 μM aprotinin. The mixture was homogenized using a Polytron PT1200 CL mixer (Kinematica, Luzern, Switzerland) for 5–90 s until a homogenous solution was obtained. An aliquot of 0.2 ml of the homogenate was thereafter further diluted 10 times in extraction buffer without the protease inhibitor cocktail. Both homogenates (diluted 1:10 and 1:100, respectively) were thereafter centrifuged at 20,800 g for 30 min at 5°C and the supernatant (dilution 1:100) without particles was frozen at -27°C for later analysis. The supernatant diluted 1:10 was immediately analyzed for tryptase.

Urine was collected at home and at the clinic, and serum was collected at the clinic and frozen immediately (within 1 h of collection). Before analysis, the urine samples were diluted 11 times in a phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1% BSA, 0.1% Tween-20, 10 mM $\text{Na}_2\text{-EDTA}$ and 0.2% N-cetyl-N,N,N-trimethylammonium bromide.

Determination of Inflammation Markers

Inflammation markers were measured in urinary and fecal samples collected during days 1, 2 and 3 and in serum collected during day 1 and 2 of placebo and active challenge.

Fecal extracts, and serum and urine samples were analyzed in duplicate for EPX by means of RIA (Pharmacia Diagnostics, Uppsala, Sweden) according to Peterson and Venge [12], and the detection limit was $<3 \mu\text{g/l}$. Because total volumes of urine produced were not known, urinary creatinine levels were measured with the alkaline picrate method (Jaffé reaction), and the results are presented as the U-EPX/creatinine ratio (in micrograms per millimole).

Tryptase was analyzed in fecal extracts in duplicate using the Pharmacia UniCAP[®] system as described by the manufacturer (Pharmacia Diagnostics). The measuring range of the assay was from <1 to 200 $\mu\text{g/l}$.

LTE_4 was determined in the urine using a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, Mich., USA). The measuring range was 8–1,000 ng/l. Samples were analyzed in duplicate according to the manufacturer's instructions, and levels were adjusted for creatinine, as above.

Eotaxin was assessed in the serum in duplicate using an ELISA (Quantikine[®] human eotaxin/CCL11 immunoassay, R&D Systems, Minneapolis, Minn., USA). The measuring range was between 15.6 and 1,000 ng/l and the detection limit was $<5 \text{ ng/l}$.

Determination of IgE Antibodies in Serum

Food-specific IgE antibodies against a mix of common food allergens (fx5; egg white, milk, fish, wheat, peanut and soya bean), wheat, milk, pork and beef and against a mix of common inhalation antigens (Phadiatop), timothy pollen and wheat pollen were

measured with the Pharmacia CAP system according to the manufacturer's instructions (Pharmacia Diagnostics).

Skin Prick Test

SPT for common food allergens was performed with food allergens according to the standard panel (milk, wheat, rye, egg, beef, pork, chicken, cod, prawn, soy, peanut and hazelnut; ALK, Hørsholm, Denmark), and the reactions were graded according to the recommendations of the Standardization Committee of the Northern Society of Allergology [24]: wheel reactions corresponding to the 10 mg/ml histamine reference were recorded as +++; double this size +++, half the histamine reference size was recorded as ++ size and half of that as +.

Statistical Analysis

Averaged values of symptom scores were calculated as mean values. Statistical analyses were carried out using Statistica[®] on a PC computer. The Wilcoxon matched pairs test and the Mann-Whitney U test for unpaired data were applied. Correlation was calculated using Spearman's correlation. The level for significance was set at $p < 0.05$.

Results

Symptoms during Challenge

Four patients out of 11 developed an increase in abdominal pain during the active challenge period. Another 4 patients reported more abdominal pain during the placebo dose. Moreover, gastrointestinal symptoms were absent in 3 patients during both placebo and active challenge.

Two of the 4 patients with symptoms to wheat and a positive DBPCFC had specific IgE antibodies against wheat pollen, and both patients had a case history of sensitivity during the pollen season. Three allergic patients and 3 non-allergic patients fulfilled the IBS criteria.

Levels of EPX Feces, Urine and Serum

The allergic patients had on the whole higher fecal levels of F-EPX compared to controls ($p = 0.043$), tended to have higher F-EPX levels throughout the study than non-allergic patients and reached significance during the active challenge period on day 15 (fig. 2). Healthy controls had F-EPX levels comparable to non-atopic patients. The highest F-EPX levels (1.14–4.61 $\mu\text{g/g}$) were observed in 1 patient with a history of food hypersensitivity and proctitis (data not shown). This patient reported higher levels of abdominal distention, pain and more frequent stools already at placebo challenge. In contrast to F-EPX, the levels of EPX in serum and urine were not increased in allergic subjects compared to non-allergic or control subjects. Fecal tryptase was only detectable in 2 patients. The

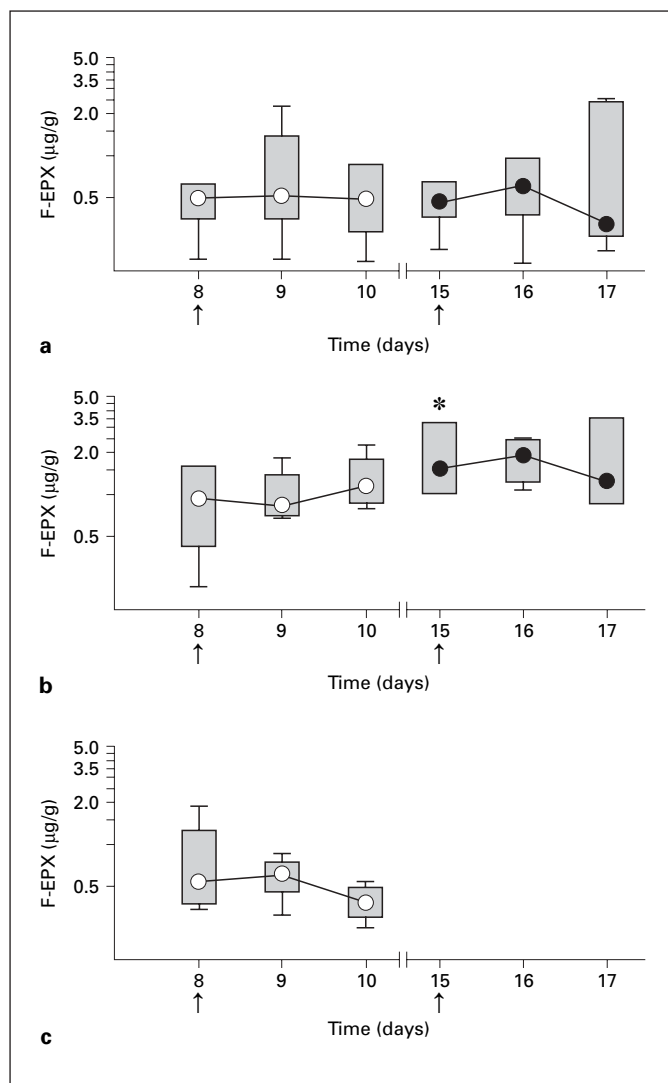


Fig. 2. F-EPX levels during placebo (○) and active challenge (●) in non-allergic patients (**a**, $n = 6$), allergic patients (**b**, $n = 4$) and control subjects (**c**, $n = 4$) are presented as medians, quartile ranges and ranges. The first sample on the 2 challenge days was collected before challenge (arrows). * $p < 0.05$, allergic vs. non-allergic patients.

highest levels were observed in the patient with proctitis (6–30 ng/g) and the pattern was similar to that of F-EPX (data not shown).

Levels of LTE_4 in Urine

The urinary levels of LTE_4 were on the whole significantly higher in allergic patients compared to non-allergic patients ($p = 0.019$), and the difference was most pronounced during active challenge (fig. 3).

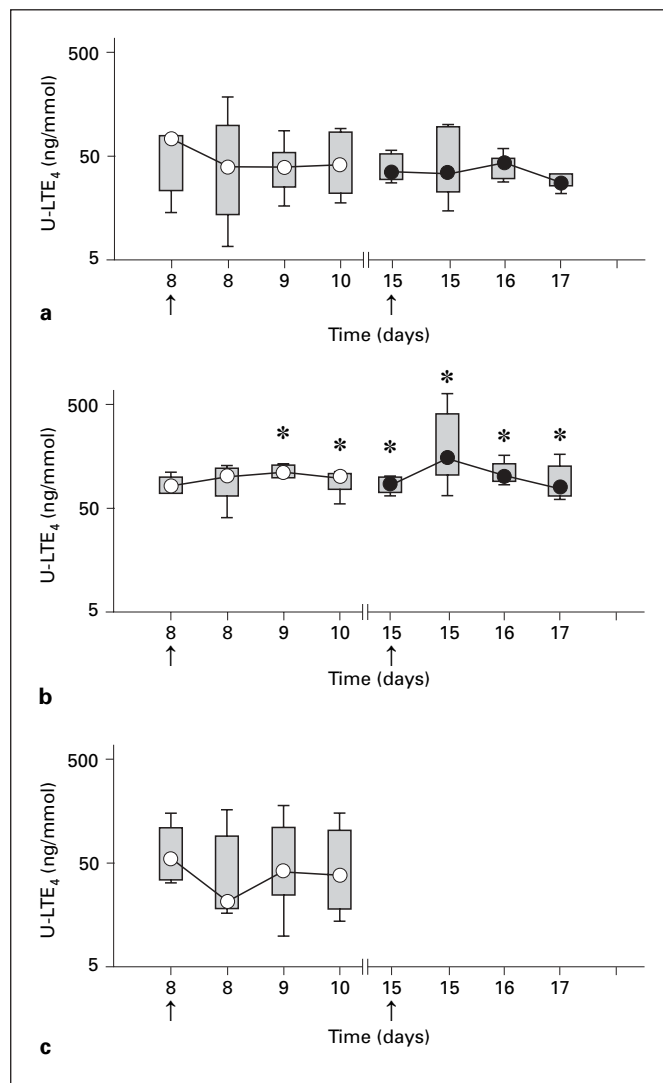


Fig. 3. U- LTE_4 levels during placebo (○) and active challenge (●) in non-allergic patients (**a**, $n = 6$), allergic patients (**b**, $n = 4$) and control subjects (**c**, $n = 4$) are presented as medians, quartile ranges and ranges. The first sample on the 2 challenge days was collected before challenge (arrows). * $p < 0.05$, allergic vs. non-allergic patients.

Levels of Eotaxin in Serum

The serum levels of eotaxin, in contrast to F-EPX and U- LTE_4 , tended to be lower in allergic patients compared to non-allergic patients and control subjects (fig. 4).

Correlations between Markers

In all samples, median levels of F-EPX correlated with U- LTE_4 levels ($R_s = 0.758$, $p = 0.011$, fig. 5). There was also a correlation between F-EPX and U- LTE_4 during placebo

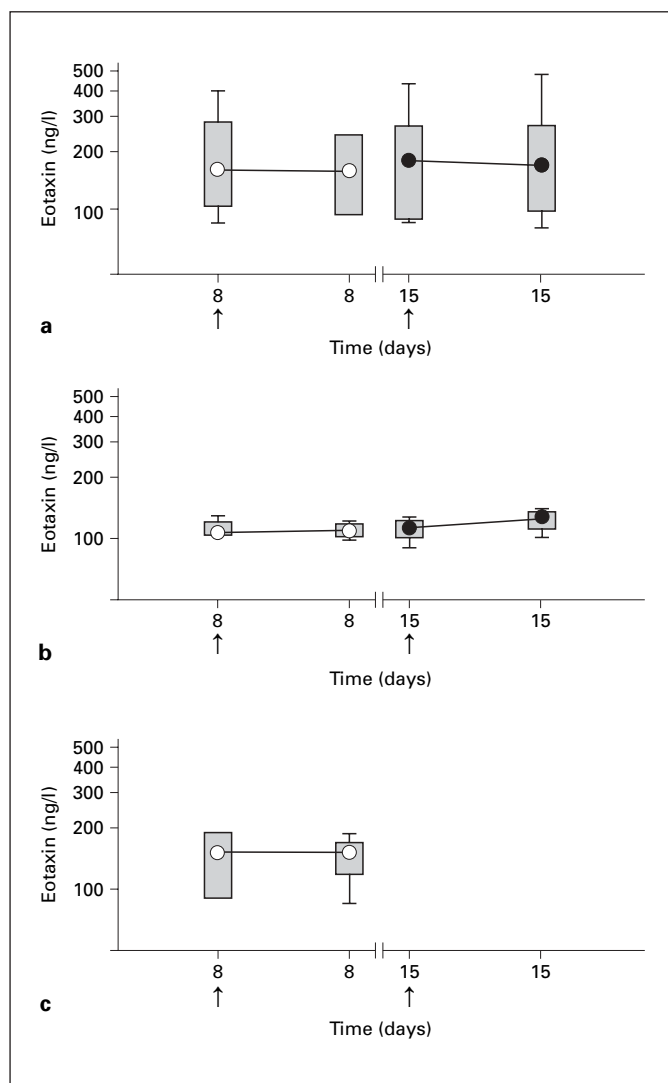


Fig. 4. Serum levels of eotaxin during placebo (○) and active challenge (●) in non-allergic patients (**a**, $n = 6$), allergic patients (**b**, $n = 4$) and control subjects (**c**, $n = 4$) are presented as medians, quartile ranges and ranges. The first sample on the 2 challenge days was collected before challenge (arrows).

challenge ($p = 0.04$) and active challenge ($p = 0.011$). However, no relationship was observed between clinical symptoms and the levels of inflammation markers.

Discussion

Food hypersensitivity with symptoms in the gastrointestinal tract is difficult to diagnose, and no perfect tool is available to support the diagnosis. Thus, several studies

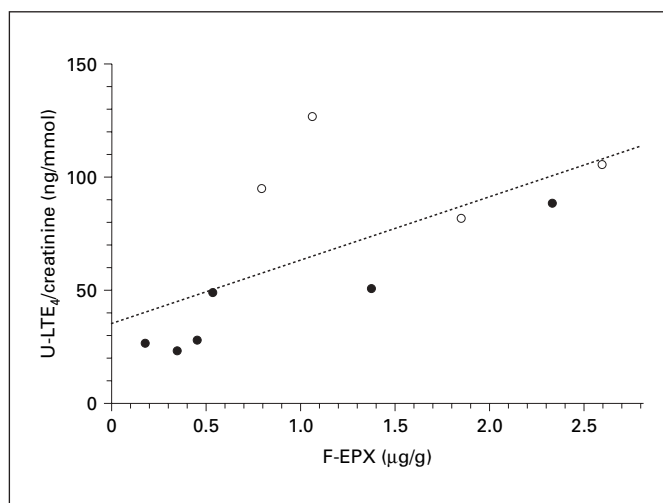


Fig. 5. Correlation between median levels of U-LTE₄ and F-EPX. ○ = Allergic patients ($n = 4$); ● = non-allergic patients ($n = 6$). $r = 0.65204$; $R_s = 0.758$, $p = 0.011$.

have demonstrated that normal values of IgE antibodies against food antigens in skin or serum do not exclude an intestinal food allergy [25, 26]. In the present study, we aimed to further investigate the role of inflammation markers in a single provocation model. We observed increased levels of F-EPX and U-LTE₄ in allergic patients compared to non-allergic patients and healthy controls. This is in agreement with our previous report that patients with food allergy and/or IBS might have a low-grade inflammation in the gastrointestinal tract, indicated by increased basal fecal levels of EPX and tryptase [15]. Furthermore, in agreement with our results, Lin et al. [10] observed increased eosinophil numbers in duodenal mucosa of patients with food hypersensitivity after 2–3 months of elimination diet, even in asymptomatic patients. In the present study, the sensitivity of the inflammation markers to detect immediate reactions after a single challenge was low. However, the increased levels of inflammation markers at the start of the study could explain the difficulties to discriminate between the effect of placebo and active challenge, at least in the allergic patients.

Interestingly, serum levels of eotaxin were decreased, and F-EPX and U-LTE₄ were increased in the same cohort of patients. Since eosinophils are the primary target for eotaxin, inducing migration of the cell in the tissue [27], the low serum levels of eotaxin may reflect eotaxin consumption by receptor-ligand reactions on eosinophils [28]. Our results may imply that the eosinophil is the ma-

major source of cysteinyl leukotrienes in the allergic inflammation of these patients since we observed a correlation between individual F-EPX and U-LTE₄ levels. This is supported by previously published data [29–31], where Macfarlane et al. [31] showed a close correlation between the number of eosinophils and cysteinyl leukotrienes in the sputum of asthmatic patients.

Six of 11 patients were regarded as suffering from IBS according to Rome II criteria and they were evenly distributed among allergic and non-allergic patients. Low-grade inflammation involving mast cells has previously been observed in some patients with IBS [14, 32]. However, in the present study, we failed to detect an increased local mast cell activity in the IBS patients as well as in most of the other patients. The patient with less severe IBD and proctitis had higher levels of F-EPX than the food-allergic patients and had also increased levels of fecal tryptase. The magnitude of the EPX levels in this proctitis patient was in complete agreement with published data [13].

Discrepancies between clinical symptoms and positive tests are often seen in adult patients, and in the present study, only 4/11 DBPCFC-positive patients were either SPT positive or had specific serum IgE antibodies against the tested food. This observation is in agreement with recent data showing that patients with positive colonoscopic allergen provocation tests often had negative SPT and low or no levels of specific IgE antibodies [26].

There was no clear discrepancy in symptoms between active and placebo challenge, and some of these patients had more symptoms after the placebo challenge, despite using a hypoallergenic formula. One could however not

exclude that a volume of the challenge drink of 300–500 ml and the lowered temperature of the drink may act as a trigger for some of the patients. Indeed, neurological disturbances in the enteric system such as abnormal gastrointestinal motility and visceral hypersensitivity are a common feature of IBS patients [33]. Thus, we cannot exclude such non-immunologic mechanisms besides a direct effect of the challenge food exerted in the IBS patients. Furthermore it is plausible that for at least some patients, a single challenge may not be sufficient for initiating a reaction, which is supported by earlier investigations where repeated challenges were required to initiate symptoms [34]. It seems therefore difficult to develop a challenge method that is suitable for all patients with food hypersensitivity.

Due to the inconsistent pattern of symptoms after placebo and active food challenge in the present study, it was not possible to relate the levels of inflammation markers to symptomatic signs of immediate reactions after food challenge. Thus, the present findings warrant further investigation of the mechanisms in food hypersensitivity in larger groups of patients. However, our data strongly indicate that activated eosinophils are involved in local reactions in the gastrointestinal tract of food-allergic patients.

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