ORIGINAL ARTICLE

Detection of Local Mast-Cell Activity in Patients With Food Hypersensitivity

CGB Peterson,[1,2] T Hansson,[2,3] A Skott,[4] U Bengtsson,[5] S Ahlstedt,[4,6] J Magnusson[5,7]

[1] Department of Medical Sciences, Clinical Chemistry, Uppsala University, Uppsala, Sweden
[2] Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden
[3] Department of Medicine, Rheumatology Unit, Karolinska Institute, Stockholm, Sweden
[4] Phadia AB, Uppsala, Sweden
[5] Asthma and Allergy Research Group, Department of Respiratory Medicine and Allergy, Sahlgrenska University Hospital, Gothenburg, Sweden
[6] Institute of Environmental Medicine, Center for Allergy Research, Karolinska Institute, Stockholm, Sweden
[7] Department of Rheumatology and Inflammation Research, Sahlgrenska University Hospital, Gothenburg, Sweden

Abstract

Background: Mast cells play a central role in many inflammatory diseases and assessment of their activation may be of use to provide objective confirmation of the outcome of food challenge in the diagnosis of food hypersensitivity. However, to date, assessment of mast-cell activation using serum markers has been unsuccessful.
Objective: The aim of this study was to explore whether locally released tryptase could be detected in stool samples from patients with food hypersensitivity.
Methods: Nine patients (median age, 55 years; range, 26 - 68 years) with food hypersensitivity confirmed by double-blind placebo-controlled food challenge were included in the study. Tryptase concentration was assessed in stool samples collected before and after an open food challenge at home and symptoms were recorded throughout the study. Tryptase concentration was also assessed in stool samples from 16 apparently healthy individuals (median age, 44 years; range, 27 - 72 years).
Results: Measurement of fecal tryptase levels in 16 healthy control subjects revealed an upper limit of the normal range (mean + 2 SD of log transformed data) of 10 ng/g. Fecal tryptase levels exceeded 10 ng/g in 7 out of 9 patients in one or more samples obtained during the study. The tryptase levels varied between patients in response to the food challenge and the individual mean levels of tryptase correlated with the corresponding levels of the inflammatory marker eosinophil protein X ($\rho = 0.7500$, $P = .02$).
Conclusion: Measurement of tryptase levels in stool samples is feasible using the method described here. Our results revealed elevated concentrations of fecal tryptase in patients with food hypersensitivity. However, several factors, including food exposure, may account for the increase in fecal tryptase and further studies are necessary to elucidate the role of mast cells in food hypersensitivity.

Key words: Asthma. Mast cell. Tryptase. Eosinophil. EPX. Food hypersensitivity. Gastrointestinal.

Resumen

Antecedentes: Los mastocitos juegan un papel fundamental en muchas enfermedades inflamatorias y la valoración de su activación puede utilizarse para proporcionar una confirmación objetiva del resultado de la prueba de provocación con alimentos en el diagnóstico de hipersensibilidad a los mismos. No obstante, hasta la fecha, la valoración de la activación de mastocitos mediante marcadores séricos no ha dado resultado.
Objetivo: El objetivo del estudio fue averiguar si la triptasa liberada localmente podía detectarse en muestras fecales de pacientes con hipersensibilidad a alimentos.
Métodos: Participaron en el estudio nueve pacientes (media de edad: 55 años; rango: 26 - 68 años) con hipersensibilidad a alimentos confirmada mediante pruebas de provocación con alimentos, controladas con placebo y a doble ciego. Se evaluó la concentración de triptasa en las muestras fecales recogidas antes y después de la provocación alimentaria en casa y se registraron los síntomas durante la realización del estudio. También se evaluó la concentración de triptasa de las muestras fecales de 16 sujetos aparentemente sanos (media

de edad: 44 años; rango: 27 - 72 años).
Resultados: La medición de los niveles de triptasa fecal en 16 sujetos control sanos reveló un límite superior de rango normal (media + 2 desviación estándar de datos transformados) de 10 ng/g. Los niveles de triptasa fecal excedieron los 10 ng/g en 7 de los 9 pacientes en una o más de las muestras obtenidas durante la realización del estudio. Los niveles de triptasa fueron variables entre los diferentes pacientes, en respuesta a la provocación alimentaria; los niveles medios de triptasa se correlacionaron con los niveles del mediador inflamatorio proteína X del eosinófilo (ρ= 0,7500, P = 0,02).
Conclusión: La medición de los niveles de triptasa en las muestras fecales es factible utilizando el método aquí descrito. Nuestros resultados revelaron concentraciones elevadas de triptasa fecal en pacientes con hipersensibilidad alimentaria. No obstante, pueden influir en el aumento de la triptasa fecal diversos factores, además de la exposición alimentaria, y son necesarios más estudios para dilucidar el papel de los mastocitos en la hipersensibilidad alimentaria.

Palabras clave: Asma. Mastocito. Triptasa. Eosinófilo. EPX. Hipersensibilidad alimentaria. Gastrointestinal.

Introduction

The lack of reliable tests for evaluating food-related gastrointestinal complaints is a significant clinical problem. Although many food hypersensitivity reactions involve immunoglobulin (Ig) E-mediated responses, it is well known that measurement of specific IgE in serum is often of limited value for the diagnosis of food allergy [1-4]. This is especially true when only abdominal symptoms occur, due to the broad range of differential diagnoses. At present, the best method for confirming the diagnosis of food hypersensitivity is the double-blind placebo-controlled food challenge (DBPCFC) [5]. Since DBPCFC requires considerable resources and is sometimes difficult to interpret due to the subjective nature of the symptoms [6-8], most studies include open dietary elimination or challenge designs [9].

Human mast cells function as effector cells in immediate hypersensitivity reactions and are widely distributed throughout the body, especially at sites close to the external environment, like the skin and respiratory tract [10]. Mast cells are also situated in the tissue proximal to the lumen throughout the gastrointestinal tract, predominantly in the lamina propria and submucosa [11]. Mast cells have long been implicated in the pathogenesis of allergic diseases and several in vivo studies indicate that they are involved in causing gastrointestinal allergy and subsequent symptoms [12,13]. Stimulation of mast cells with IgE-dependent or non-IgE-dependent agonists leads to the release of preformed and newly synthesized inflammatory mediators, such as histamine, proteases, leukotrienes, prostaglandins, and cytokines [10]. Measurements of in vivo release of mast cell mediators might therefore reflect both true gastrointestinal food allergy and nonallergic food hypersensitivity reactions, and provide more objective confirmation of the outcome of food challenge [13].

Tryptase, which is released almost exclusively by mast cells, could be suitable as a marker of mast-cell activation in patients with food hypersensitivity [10]. However, Sampson et al [14] and Vila et al [8] failed to detect tryptase in serum samples from patients who had a positive reaction to DBPCFC or in patients with food-induced anaphylaxis. This failure to detect tryptase could be due to predominantly local secretion from mast cells residing in the tissue. In this study, we developed a method for measurement of tryptase in fecal samples as a marker of mast cell activity in the gut. We then used this technique to analyze fecal tryptase in healthy subjects and food-hypersensitive patients before and after food challenge.

Material and Methods

Patients

Nine patients with food-related gastrointestinal symptoms were included in the study (median age, 55 years; range, 26-68 years). The clinical characteristics of the study population are summarized in the table. Two patients (numbers 7 and 8) presented an IgE-mediated food allergy to wheat and pork, respectively, whereas the remaining patients were considered to have nonallergic food hypersensitivity according to World Allergy Organization criteria [15]. All patients were confirmed positive by DBPCFC at the Department of Respiratory Medicine and Allergy, Sahlgrenska University Hospital, Gothenburg, Sweden and then underwent an open food challenge with time-course analysis at home for 6 weeks outside the pollen season to study gastrointestinal eosinophil and neutrophil inflammation, as described previously [16].

The patients were instructed to eliminate suspect food from their diet for 2 weeks and then to consume (challenge) the suspect food during the third week. During the remaining 3 weeks of the study, the patients followed an elimination diet (Figure 1). All patients were instructed to register their intake of the suspect food during the 7 days of open challenge and to record their abdominal symptoms throughout the study. The patients collected stool samples every day during weeks 2 to 4 and once at the ends of weeks 5 and 6. The presence of celiac disease was ruled out in all patients according to international criteria [17], whereas 5 patients fulfilled the ROME II criteria for irritable bowel syndrome (IBS) [18].

Stool samples were also obtained from 16 apparently healthy individuals (median age, 44 years; range, 27-72 years). None of those individuals had a history of allergy, vascular disease, or diseases of the lungs, gastrointestinal system, thyroid, heart, liver, or joints and none had current infectious disease, dermatitis or eczema, diabetes, or tumors or were receiving anti-inflammatory treatment.

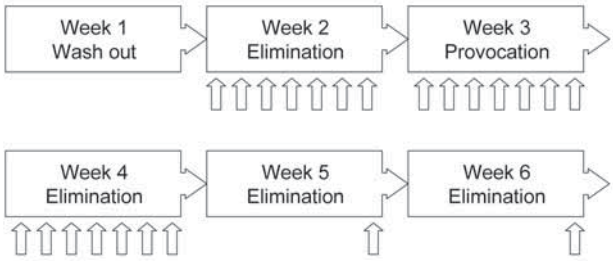

Figure 1. Study design. Arrows indicate days on which fecal samples were collected—once daily in weeks 2, 3, and 4, before, during, and after challenge, respectively, and then once weekly in weeks 5 and 6, after challenge.

Stool Preparation

After collection, stool samples were immediately frozen and kept at —20°C until analyzed. Processing of stool samples was done as described previously [19], with some modifications. Briefly, fecal samples were kept on ice and diluted in various volumes of an extraction buffer consisting of phosphate-buffered saline supplemented with 12 mM ethylenediamine tetraacetic acid, 0.1% N-Cetyl-trimethyl ammonium bromide (CTAB), 20% glycerol, 0.005% Tween 20, 1% bovine serum albumin, and a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, USA) containing 4 mM 4-(2-Aminoethyl)-benzensulfonyl fluoride (AEBSF), 0.26 mM Bestatin, 28 μM E-28, 2 μM Leupeptin, and 0.6 μM Aprotinin, pH 7.4. The mixture was then homogenized using a Polytron mixer until a homogenous solution was obtained (approximately 10-90 seconds). The homogenate was centrifuged at 20 000 g for 30 minutes at 4˚C and the supernatant was collected for analysis. The final sample treatment procedure included 10-fold dilution of approximately 0.2 g (approximately 0.2 mL) of a fecal sample with 9 volumes of extraction buffer.

To adjust for fecal water content, the pellet obtained after centrifugation was weighed and the weight of the pellet (semidry weight) was divided by the weight of the unprocessed sample to obtain an adjustment factor. The calculated fecal concentrations of tryptase (ng/g) and eosinophil protein X (EPX) (μg/g) were obtained by dividing the measured concentrations (μg/L) by the adjustment factor. Tryptase used for spiking experiments was purified to homogeneity from human lung as previously described [20].

Analysis of Tryptase in Stool

Tryptase concentration was analyzed by immunoassay performed in duplicate using the ImmunoCAP system (Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. The measurement range of the assay was <1 to 200 μg/L and the coefficients of variation based on 3 control samples analyzed in duplicate on 18 occasions were between 1.8% and 3.6% within assays and between 6.4% and 10% between assays. The fluorescence of the samples was always adjusted for the fluorescence of the diluent before evaluation.

Analysis of EPX in Stool

EPX concentration was analyzed using a commercial radioimmunoassay (Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. Concentrations of EPX of up to 1.7 μg/g were considered normal [19].

Analysis of Serum Specific IgE Concentration

Food-specific IgE concentration was measured by immunoassay using the Pharmacia CAP system (Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions.

Skin Prick Test

Skin prick test (SPT) was performed with a standard panel of food allergens and inhaled allergens (ALK, Hörsholm, Denmark): milk, wheat, rye, egg, beef, pork, chicken, cod, prawn, soy, peanut, hazelnut, house dust mite (*Dermatophagoides pteronyssinus and D farinae*), mold (*Aspergillus fumigatus*), dander (dog, cat, and horse), grass (*Phleum pretense and Dactylis glomerata*), herb (*Artemisia vulgaris*), and tree (Betula). SPT was performed according to the recommendations of the Standardization Committee of the Northern Society of Allergology [21]. Wheal reactions of a size corresponding to that obtained with 10 mg/mL histamine were graded as +++; wheals twice that size, ++++; wheals half the histamine reference size, ++; and wheals a quarter of the histamine reference size, +.

Statistical Analysis

Statistical analyses were carried out using Statistica software (Statsoft Inc, Tulsa, USA). The Wilcoxon matched pairs test and Mann—Whitney *U* test for unpaired data were applied and correlations assessed using the Spearman correlation coefficient. Statistical significance established at $P < .05$.

Results

Protease Inhibitors Increase the Recovery of Tryptase in Feces

Initial spiking experiments indicated low recovery of tryptase in fecal samples (< 41%), irrespective of the buffers used for extraction. The presence of detergents like CTAB together with glycerol and a cocktail of protease inhibitors dramatically increased the recovery of tryptase. Optimization of the protease inhibitor concentration with spiked fecal samples from 2 healthy individuals showed a dose-dependent effect of protease inhibitors on recovery of tryptase (Figure 2). A maximal effect of protease

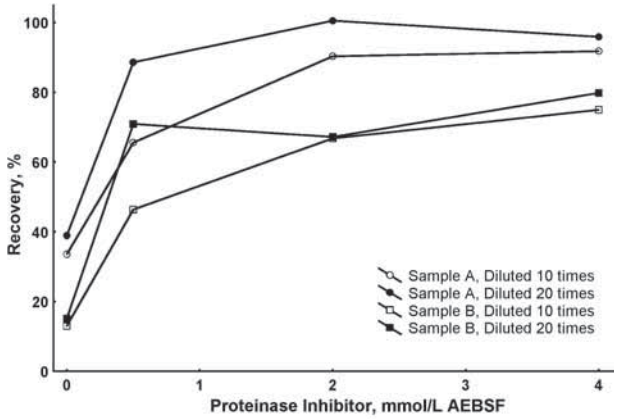

Figure 2. Tryptase recovery after addition of protease inhibitors. Two fecal samples were spiked in triplicate with tryptase and diluted as indicated with extraction buffer containing protease inhibitor cocktail at a final concentration of 0, 0.5, 2, and 4 mmol/L 4-(2-Aminoethyl)-benzensulfonyl fluoride (AEBSF), with all other components at the same relative dilution.

inhibitors on recovery was observed at a 50% dilution of the stock mixture, corresponding to an AEBSF concentration of 2 mM (all other components diluted accordingly). Slightly better recovery was observed at a 1:20 dilution of the fecal sample compared to that with a 1:10 dilution, especially at lower concentrations of protease inhibitors. However, given that these experiments indicated nearly undetectable levels of tryptase in fecal samples, a 10-fold dilution of the sample in extraction buffer was preferred in order to extend the ability to detect tryptase.

The extraction buffer did not interfere with the measurement of tryptase, and spiked fecal samples from 3 individuals demonstrated that the variation observed in tryptase concentration according to extraction time was insignificant

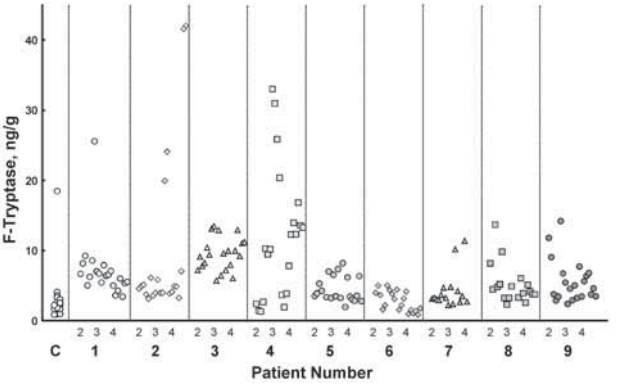

Figure 3. Tryptase concentrations in stool from healthy individuals and patients with food-induced gastrointestinal hypersensitivity. Stool samples were collected once from 16 healthy individuals (C) and from 9 patients every day during week 2 (elimination diet), week 3 (food challenge), and week 4 (follow-up).

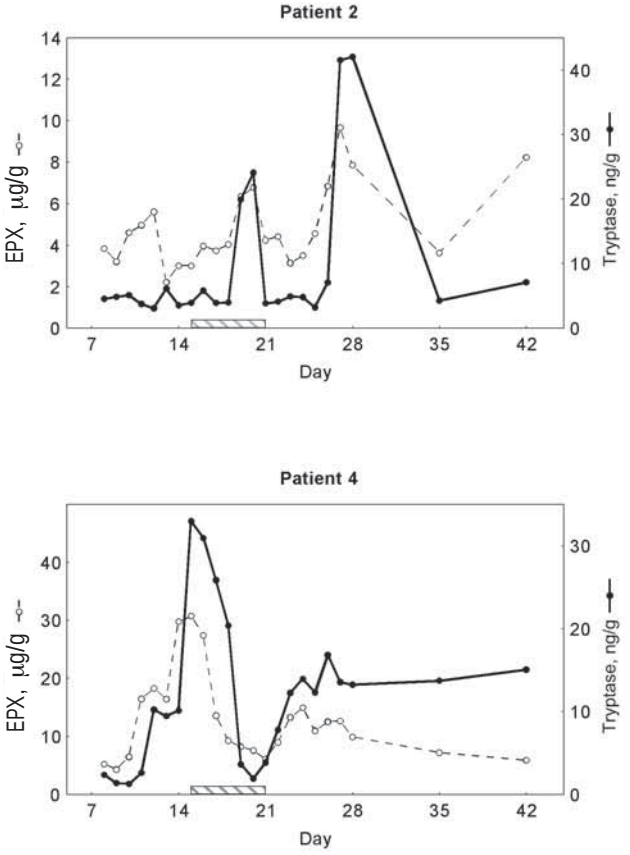

Figure 4. Increase in fecal tryptase and EPX in 2 patients with gastrointestinal hypersensitivity in response to food challenge. Fecal concentrations of tryptase (—●—, ng/g) and EPX (···○···, µg/g) in samples collected every day during week 2 (elimination diet), week 3 (food challenge), and week 4 (follow-up), and at the end of weeks 5 and 6 (follow-up)

for up to 5 hours extraction (data not shown).

Increased Tryptase Concentrations in Stool From Patients With Food Hypersensitivity

Fifteen of the 16 healthy individuals had fecal tryptase concentrations lower than 10 ng/g, which corresponds to the upper limit of the calculated normal range (mean + 2 SD of log transformed data) (Figure 3). In 7 of the 9 patients with food hypersensitivity, fecal tryptase concentrations of more than 10 ng/g were obtained on some days during the 3 weeks in which stool samples were collected daily (Figure 3). Patients 1, 8, and 9 showed the highest individual tryptase concentrations prior to challenge. All 9 patients responded to the challenge with abdominal symptoms, but the profile of fecal tryptase concentration in response to the challenge varied between patients; eg, only 2 patients (numbers 2 and 4) responded with a marked increase in tryptase concentration during the food challenge and those 2 patients also had a late-phase increase in tryptase concentration during the post-challenge week (Figure 4). Two patients (patients 7 and 8) had raised serum

Patient History and Laboratory Data of the 9 Food-Sensitive Patients Included in the Study*

Patient	Age, y/Sex	Outcome of DBPCFC	SPT for DBPCFC Food	Specific IgE for DBPCFC Food†	Onset Time of Symptoms	Symptom Duration, h	Gastrointestinal Symptoms After Challenge	SPT Inhalant Allergens	Concomitant Atopic Disease
1	68/F	Milk	-	-	1 h	12-24	AD, D	+	-
2	68/F	Milk	-	-	5 min	12-24	AD, AP, D	-	Rhinoconjunctivitis
3	64/F	Milk	-	-	5 min	12-24	AD, D,	-	-
4	56/F	Wheat	-	-	1 h	12-24	AP, D	+++	Asthma
5	55/M	Milk	-	-	1-24 h	24-48	AP, D	+	Rhinoconjunctivitis
6	44/F	Milk	-	-	24 h	24-48	FL	-	-
7	42/F	Wheat	++	4	24 h	24-48	AD, AP, D, FL	+++	Rhinitis
8	38/F	Pork	+	1	1-24 h	24-48	AP,	+++	Rhinoconjunctivitis
9	26/M	Egg	-	-	1 h	12-24	AP, C/D	-	-

*DBPCFC indicates double-blind placebo-controlled food challenge; SPT, skin prick test; Ig, immunoglobulin; AD, abdominal distension; D, diarrhea; AP, abdominal pain; FL, flatulence; C, constipation.

†Specific IgE class 4, 17.5 – <50 kU_A/L; class 1, 0.35 – 0.7 kU_A/L

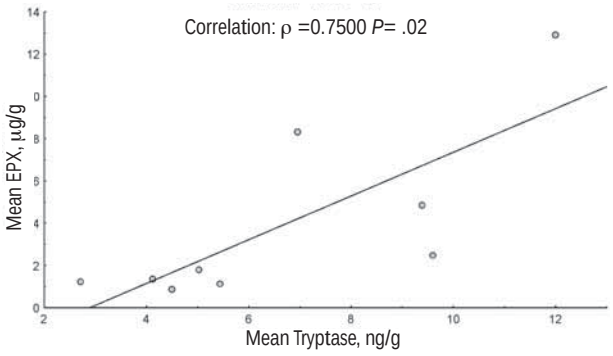

Figure 5. Relationship between fecal concentrations of tryptase and EPX. Mean values of all individual fecal concentrations of EPX were plotted against the corresponding mean values of fecal tryptase for the patients with food hypersensitivity (ρ = 0.7500, *P* = .02).

concentrations of food-specific IgE. However, those patients did not respond or showed only a weak response in terms of fecal tryptase concentration during the food challenge.

Fecal Tryptase Concentrations Correlate With Patient History and Eosinophil Markers

Five patients with a history of a short duration of symptoms (12- 24 hours) or a short onset time of symptoms (5 minutes to 1 hour) (table) had significantly higher mean levels of tryptase than 4 patients with a longer duration or longer onset time of symptoms (*P* = .014). The tryptase profile in patients 2 and 4 seemed to be very similar to that of EPX throughout the study period (Figure 4); thus, tryptase concentrations correlated with EPX concentrations in those patients (*P* = .047 and *P* = .022, respectively) and also in patient 9 (*P* = .019). Moreover, the individual mean concentrations of tryptase correlated with the corresponding concentrations of EPX (Figure 5) (ρ = 0.7500, *P* = .02).

Discussion

Several studies have indicated a central role for mast cells in various pathological conditions, including food hypersensitivity [11]. In this study, we sought to determine whether locally released tryptase could be detected in patients with food hypersensitivity. We therefore optimized the measurement of mast-cell tryptase in fecal samples in terms of extraction buffer and protease inhibitor concentration, and the validity of the measurements was verified by showing that tryptase was almost completely recovered from spiked fecal samples.

High levels of tryptase were found in 7 out of 9 patients on one or more occasions during the 3 weeks of the study, whereas increased fecal tryptase concentrations were only observed in 1 of the healthy subjects. No uniform pattern could be observed in the profile of tryptase concentration among the patients, despite the similarity of their symptoms during the food challenge. However, 2 patients (numbers 2 and 4) showed

markedly increased tryptase concentrations during the food-challenge period. This was consistent with a previous report showing a rapid increase in intestinal release of tryptase in patients with food allergy after intraluminal administration of food antigens [22]. Surprisingly, those 2 patients had no history of IgE-mediated allergy. However, we can not rule out the possibility of local production of allergen-specific IgE in the gastrointestinal tract of those patients [13].

Five of the 9 patients fulfilled the ROME II criteria for IBS and we therefore can not exclude the possibility that a mechanism other than a direct effect of the foods was operating in some of the patients. Indeed, low grade inflammation of the gut involving mast cells has been observed in subsets of patients with IBS [23] and increased concentrations of fecal tryptase have been detected in patients with IBS [24], suggesting that a subset of IBS patients could have dysfunctional mast-cell regulation. Psychological stress, which has been considered to provoke mast cell activation, may partly explain the increase in tryptase during the week prior to the challenge [25]. Furthermore, neuropeptides have been shown to be potent mast cell activators, resulting in the release of proinflammatory mediators [26]. Another possible explanation of non-IgE-mediated activation of mast cells is cell-mediated immunological reactions in response to food antigens involving activated subsets of T cells, perhaps in combination with stress [27,28].

Tryptase concentrations in stool were compared with fecal concentrations of the eosinophil marker EPX. In comparison with eosinophil cationic protein, EPX concentration is more often increased in food-hypersensitive patients [16] and patients with IBD [19]. The temporal profile of tryptase concentration was similar to that of EPX, an observation which may strengthen the validity of the present results. Moreover, this indicates that both eosinophils and mast cells are involved in the pathogenesis of food hypersensitivity. The majority of the patients with high concentrations of fecal tryptase, as well as EPX, also had a history of relatively short duration of symptoms together with a short time of onset of symptoms after challenge. This pattern of symptoms together with eosinophil activity may correspond to a response similar to that described in atopic dermatitis as a cutaneous late-phase response to allergen [29] with mast-cell activation and subsequent infiltration of inflammatory cells such as eosinophils.

Food hypersensitivity is regarded as a heterogeneous condition of variable etiology, and it is clearly possible that there are several immunological mechanisms underlying food-related gastrointestinal problems in patients with food hypersensitivity. In the present study, we showed elevated concentrations of fecal tryptase in patients with food hypersensitivity. However, we were not able to distinguish patients with IgE-mediated food allergy from those with non-IgE-mediated food hypersensitivity on the basis of tryptase concentration. Further studies are warranted to elucidate the role of mast cells in food allergy and, in particular, non-IgE-mediated food hypersensitivity.

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Dr Christer Peterson

Department of Clinical chemistry and Pharmacology
University Hospital
SE-751 85 Uppsala
Sweden
E-mail: christer.peterson@medsci.uu.se