

Analysis and Characteristics of Mast Cell Tryptase and Eosinophilic Cationic Protein from Human Gut Mucosa in Gastrointestinal Allergy

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Introduction

Food allergy (FA) may cause both intestinal and extraintestinal symptoms. Nearly all human organs have already been reported as potential shock organs in food hypersensitivity reactions including cardiopulmonary system and heart, central nervous system (brain, eye, ear, etc.), gastrointestinal tract (GI tract), kidney, joints, mucous membranes, respiratory and urogenitary tract as well as the skin [1–5]. The diagnosis of food hypersensitivity is commonly based on history, skin tests, total and specific IgE antibody determinations in serum as well as oral food challenge tests. However, in gastrointestinal allergy (GA) food-specific sensitization may be confined only to the intestine causing primarily unspecific abdominal symptoms like diarrhea, vomiting, malabsorption, epigastric pain, abdominal cramps, bloating, etc., which may also arise from other important gastrointestinal disorders. Typical extraintestinal symptoms like asthma, flush, hypotonia, rhinitis, urticaria, etc. may also occur in GA, but only facultatively, as a consequence of either an excessive intestinal mediator release or as result of an extraintestinal binding of the ingested antigen(s) or its metabolite to organ-specific IgE antibodies.

Since the simultaneous appearance of intestinal and extraintestinal symptoms is more often highly suggestive of an underlying allergic reaction to food-stuffs both for patients and physicians than the presence of only abdominal symptoms, GA is sometimes extremely difficult to diagnose [1–4]. Such pa-

tients with abdominal complaints like chronic diarrhea, malabsorption, weight loss or bloating, etc. are primarily or naturally referred to the gastroenterologist who examines, in general, the upper and lower GI tract for underlying organic diseases. The potential spectrum of differential diagnosis to GA, however, is extremely broad [1–4]. Nearly all patients with GA present initially with unspecific abdominal symptoms and show often uncharacteristic anamnestic or laboratory findings. Endoscopy and histology of the whole GI tract is often necessary to exclude potential differential diagnosis or other rare diseases (e.g. collagenous colitis).

However, data about the prevalence and incidence of FA or GA in a population of patients presenting primarily with gastrointestinal symptoms like chronic diarrhea are very scarce in the literature. In this respect it is also still uncertain whether the routine histologic examination of the GI tract yields decisive clinical pictures in GA and whether the intestine is entirely involved or only localized parts of it. Furthermore, in such cases it has been reported that skin tests and IgE antibody determinations in serum might fail to demonstrate clear systemic signs of food-specific sensitization [1–6], making the diagnosis of allergic reactions of the intestine more difficult than in other forms of FA with extraintestinal manifestations and/or a more systemic nature of the IgE antibody response [3, 4, 6].

Since eosinophils and mast cells have been implicated for many years in the pathogenesis of allergic diseases, the objective of the present investigations was to evaluate the characteristics of mast cell tryptase (T) and eosinophilic cationic protein (ECP) in human gut mucosa of patients suffering from chronic diarrhea, malabsorption, colitis and other abdominal symptoms due to manifest GA. To more precisely define the role mast cells and eosinophils play in the lower GI tract in such patients, practical tissue homogenization and biopsy incubation methods (mucosa oxygenation) had to be constructed to allow – on the basis of tiny endoscopic samples – a quantitative extraction and most actual measurement of these mediators from gastrointestinal tissue.

Material and Methods, Patient Populations

Patients

For the investigation of the characteristics of ECP and T in the lower GI tract, a total of 35 persons [15 control group, 20 allergic enteropathy (AE)] were examined with upper and lower gastrointestinal endoscopy. All patients gave their informed consent. Age, sex, the number of patients and biopsies from each group are listed in table 1. Control patients suffered neither from intestinal polyps or cancers nor from inflammatory or allergic processes of the gut. These patients underwent nonemergency colonoscopy for (1) anemia work-up or (2) evaluation of constipation, (3) abdominal pain, (4) diverticular disease or (5) perianal bleed-

Table 1. Mucosal ECP and T content of human gut mucosa and their rates of spontaneous, anti-IgE- and allergen-induced secretion from intact endoscopic samples processed by mucosa oxygenation

Group	Patients n	Sex f/m	Age	Biopsies (total number)	Mediator release, ng mediator/ mg wet weight	Tissue mediator con- tent, ng mediator/ mg wet weight
<i>Eosinophilic cationic protein (ECP)</i>						
Control group	15	7/8	52	122	2.7 ± 1.1	22.4 ± 29.6
Induced ECP release						
Anti-IgE				42	5.3 ± 1.0	
Allergic enteropathy: manifest disease, without any treatment	20	6/14	38	112	4.5 ± 4.1	56.5 ± 52.1 ^a
Induced ECP release						
Anti-IgE				46	12.8 ± 6.8	
Allergic enteropathy: remission, allergen elimination diet	4	2/2	44	38	–	33.3 ± 21.1
<i>Mast cell tryptase (T)</i>						
Control group	15	7/8	52	119	2.9 ± 1.6	33.3 ± 21.1
Induced T release						
Anti-IgE				28	3.7 ± 3.1	
Allergic enteropathy: manifest disease, without any treatment	20	6/14	38	112	3.6 ± 1.7	61.0 ± 41.7 ^b
Induced T release						
Anti-IgE				36	5.9 ± 6.4	
Allergic enteropathy: remission, allergen elimination diet	4	2/2	44	38	–	46.7 ± 31.2

Significance levels (compared to control group): ^ap < 0.001; ^bp = 0.001.

ing. All persons of this group had grossly and histologically inconspicuous findings of the colon and rectum with the exception of incidental hemorrhoids.

Patients complaining of gastrointestinal symptoms (abdominal pain, diarrhea, bloating, vomiting, etc.) underwent – among many other diagnostic tests (H₂ breath tests, stool cultures for infectious agents and exocrine pancreas insufficiency, gastroscopy including duodenal and stomach biopsy, etc.) – finally colonoscopy to exclude other causes of gastrointestinal disease like inflammatory bowel disease, collagenous colitis or mastocytosis, etc. Among patients with GA, extraintestinal symptoms were present in only 20–35% and occurred either spontaneously or in a more or less severe association with the ingestion of certain meals. At the occasion of diagnostic colonoscopy, histologic examinations and tissue mediator measurements were made routinely to screen for patients with mucosal eosinophilia or abnormal

mediator levels. In patients without significant objective or unspecific findings, but persistent chronic or intermittent diarrhea and/or other persistent gastrointestinal complaints, a special diagnostic program was additionally performed to look for AE by anamnesis, detailed prick skin tests (molds, fibers, bacteria, pollens, dust, fish, fruit, vegetables, grains, flour, brans, milk, egg, cheese, tea, coffee) and by specific IgE determinations (RAST). In each patient the diagnosis of FA was clinically confirmed by double-blind, placebo-controlled oral food challenge (DBPCFC) with the suspected food antigen(s) or a panel of standard antigens (cheese, egg, fish, fruits, flour, grains, meat, milk, nut, spices, tea and coffee, vegetables). DBPCFC was performed with freshly prepared foodstuffs which were for the most part homogenized and mixed (masked) with a liquid oligopeptide formula. The test solutions as well as placebo were administered to the patient by a nasogastric tube inserted in the proximal stomach under radiological conditions. Clinical reactions (subjective, objective, laboratory data) were recorded by a standardized scoring system and positive reactions were reproduced in more than 90% by a second blinded challenge.

In addition to this, in 9 of these patients colorectal biopsies were incubated with allergen(s) (cheese, barley, rye, soya or wheat flour, milk, nuts, pork, spices) or control food antigens (Bencard, Germany; Hollister-Stier, Germany) to compare the rate of mucosal ECP secretion in response to these agents with the outcome of DBPCFCs. For this comparison, a mediator release which should elicit a clinically relevant adverse reaction on DBPCFC was defined 'as significantly increased' when exceeding the double of the spontaneous ECP release.

Mucosa Oxygenation: Mediator Release from Human Gut Mucosa

The endoscopically obtained mucosal samples were immediately (within 10 s) placed into hard plastic tubes (Nunc) with 4,000 μ l of a modified Hanks' incubation medium (supplemented with human serum albumin, PIPES, HEPES buffer and fetal calf serum) that was bubbled with a steady flow of room air to ensure sufficient oxygen pressures inside the biopsy [7-9]. Mucosa oxygenation (37°C, pO_2 85-100 mm Hg, pH 7.4-7.6) prevented the occurrence of ischemic conditions for the mucosal tissue [7, 9, 10]. To ensure an accurate mediator measurement (e.g. histamine, T) when tissue particles are used, it is necessary to strictly avoid ischemic conditions, since ischemia in general - but particularly and highly effective in the gut mucosa - causes an unproportionally high rate of histamine degradation by naturally occurring enzymes (e.g. diamine oxidase) and a disturbed mediator response [7, 10-12].

The biopsies from 1 patient were separately used for measurement of the spontaneous mediator release (2 samples) and the stimulated release of ECP and T in response to anti-IgE (Sigma, Germany; optimum dilution 1:40 v/v; 2 samples) or allergens (Bencard; optimum dilution 1:10,000 v/v; 4-6 samples). Mucosa oxygenation was performed during the whole incubation time of 4 h. At 2.5, 5, 7.5, 15, 30, 60, 120, 150, 180, 210 and 240 min a volume of 400 μ l of the supernatant was removed and in each case the released amount of ECP and T calculated for the actual volume by a GW-BASIC software program. At the time point of 60 min and later the volume of the incubation medium was each corrected to 4 ml by addition of pure incubation medium without stimulus. The net mediator release (ng mediator/mg wet weight, mean $\pm$ SD) resulted from addition of the increase in mediator amounts released during the whole incubation time [9].

For the investigation of the total tissue ECP and T content, biopsies were additionally taken from the same areas of the lower GI tract as for the release experiments, but these samples were immediately put into liquid nitrogen (-72°C) until performance of the mechanical homogenization (Micro-Dismembrator, Braun-Biotech, Germany) to avoid any mediator degradation. One tissue sample was homogenized by three homogenization steps using (A) 1,500 μl aqua bidestillata (1,500 rpm, 5 min), (B) 1,500 μl aqua bidestillata (1,500 rpm, 3 min) and (C) 1,500 μl protein-containing incubation medium (1,500 rpm, 3 min). Homogenization steps B and C were performed to wash the homogenization tube (from A), since former studies showed a mediator binding to plastic tubes. The net mediator content resulted from addition of the mediator amounts detected in A–C and is expressed in ng mediator/mg wet weight (mean $\pm$ SD). Biopsy wet weights were detected by the use of a microbalance (Sartorius, Germany).

ECP and T Measurement: Assay Characteristics and Statistical Analysis

ECP and T measurements were made by specific radioimmunoassays (Pharmacia, Sweden) according to the manufacturer's instructions. Intra- and interassay variations of the ECP RIA were 10.7 and 18.8% for supernatants of the incubation medium and 15.5 and 15.9% for tissue homogenates, respectively. Intra- and interassay variations of the T RIA were 4.7 and 7.8% for supernatants of the incubation medium and 10.4 and 14.6% for tissue homogenates, respectively. For statistics, comparisons were each made using the U test (Wilcoxon, Mann-Whitney); significance levels are given in the tables or figures.

Results

Tissue ECP and T levels (mean $\pm$ SD) of the lower GI tract are listed in table 1. These results do not only show a significant accumulation of ECP and T in human gut mucosa in AE, but they indicate also that these mediator levels may fall during an effective allergen elimination diet.

Figures 1 and 2 illustrate the distribution of ECP and T throughout the individual sections of the lower GI tract from the terminal ileum to the rectum. In healthy individuals, ECP and T levels were similarly distributed with the highest concentrations in the terminal ileum and proximal colon, which gradually decrease to the distal colon and rectum ($\text{ECP} > \text{T}$). In contrast, patients with GA were found to contain both clearly higher mediator levels and abnormally distributed mediator concentrations, which, in general, did not gradually decrease from proximal to distal. However, the relatively high variations of the tissue mediator levels may result from interindividual as well as from intraindividual variations (between different segments within 1 person) due to a more patchy distribution of these mediators within 1 person. In fact, a patchy distribution of ECP and T was observed in several patients, showing high or excessive mediator levels in only one or two sections of the large intestine, while others had inconspicuous mediator levels.

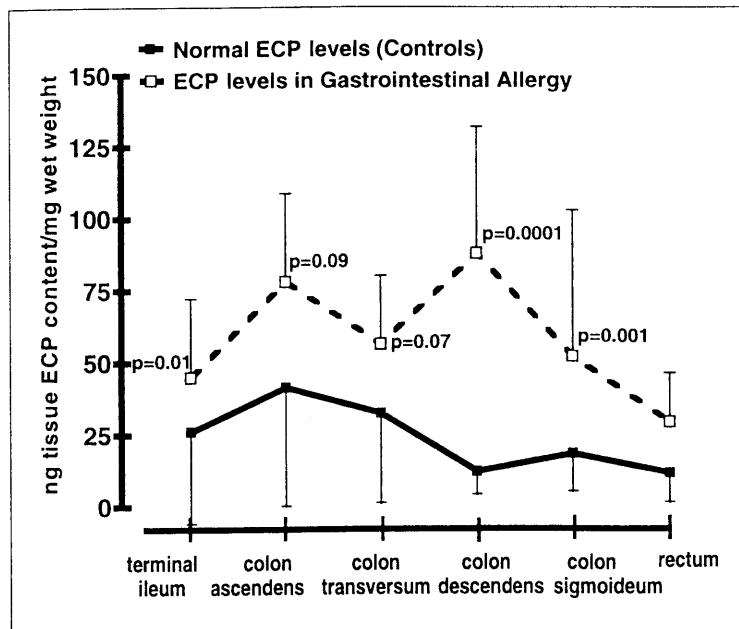


Fig. 1. Distribution of ECP throughout the lower GI tract in normal human gut mucosa and GA.

The spontaneous and stimuli-induced rates of ECP and T secretion in controls and GA are also listed in table 1. As expected, the spontaneous ECP and T secretion in patients with AE were higher than in normal gut mucosa. But when comparing ECP and T release, spontaneous ECP secretion appeared to be more pronounced than T secretion in GA.

The functional activity of intestinal eosinophils and mast cells was assessed by detection of ECP and T secretion in response to anti-IgE and allergens. As shown in table 1, both control persons and patients with GA were found to contain functionally reactive immune effector cells in gut mucosa. However, in AE (1) the release of ECP and T could be more vigorously enhanced than in controls, and again (2) ECP secretion in allergic patients exceeded clearly T secretion in these patients. In contrast to previous histamine release experiments [8], the kinetics of ECP and T secretion in response to anti-IgE increased mainly after 60 min of stimulation (data not shown), but at latter time points or at the end of the incubation period (4 h) both mediators showed each significantly enhanced rates of ECP and T secretion.

When biopsies from patients with GA were incubated with food antigens, which were proven in DBPCFC as causal allergens or tolerated antigens, both

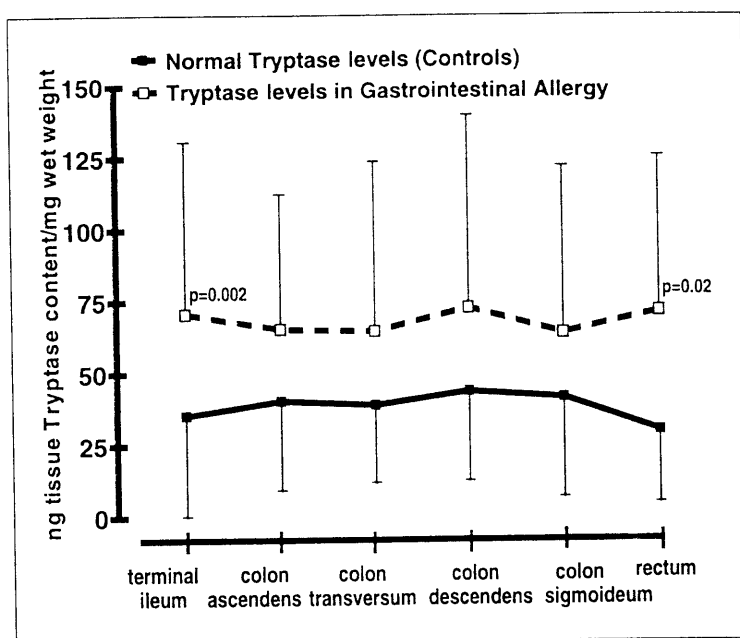


Fig. 2. Distribution of mast cell T throughout the lower GI tract in normal human gut mucosa and GA.

mediators showed significant different kinetics between DBPCFC-positive or DBPCFC-negative allergens (fig. 3, 4). In patients without food-specific IgE antibodies in serum and positive challenge reactions, intestinal washings were performed [13] to prove whether the observed mediator release was immunologically or nonimmunologically mediated. The presence of intestinal food-specific IgE antibodies could be demonstrated in 3 of 9 patients (mostly isolated from terminal ileum), which confirmed that an immunologically mediated mediator release had been induced by the corresponding antigen.

The intestinal mediator release in response to allergens was defined as 'significantly enhanced or clinically relevant' when the mediator release in response to the allergen exceeded the double of the spontaneous release. By this way, allergen-induced intestinal ECP release identified – on the basis of this preliminary study in 9 patients with confirmed GA – 75% of DBPCFC-positive antigens and 100% of DBPCFC-negative allergens (table 2), indicating that intestinal mediator release may be a useful tool to (pre)select antigens for the confirmatory DBPCFC.

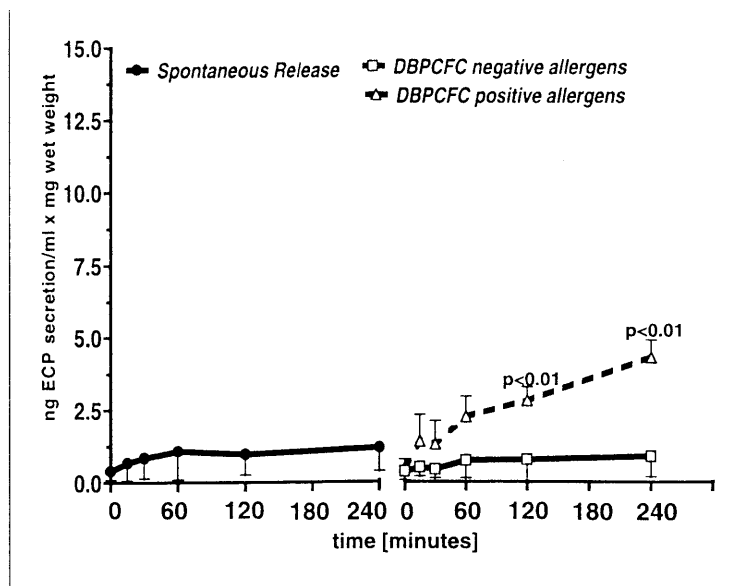


Fig. 3. Release of ECP from human gut mucosa in response to DBPCFC-positive and DBPCFC-negative allergens in 9 patients with GA.

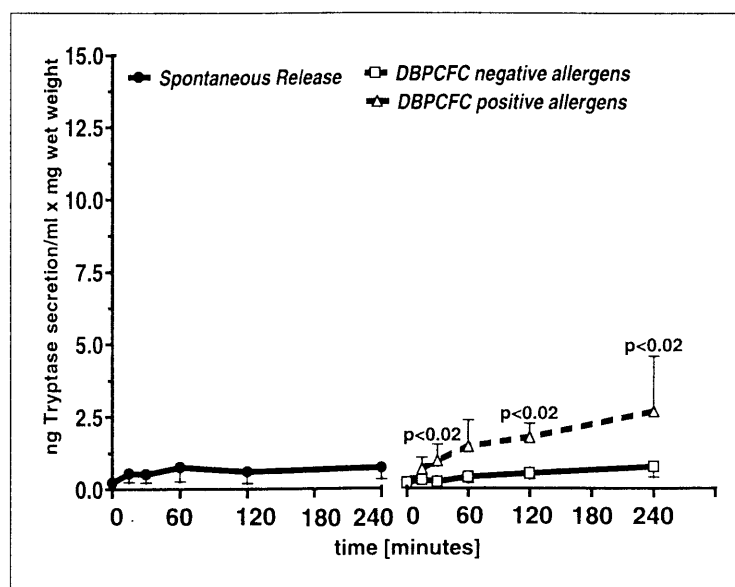


Fig. 4. Release of mast cell T from human gut mucosa in response to DBPCFC-positive and DBPCFC-negative allergens in 9 patients with GA.

Patient population	20 patients with GA (confirmed by DBPCFC)	
Patients studied with allergen-induced ECP release and subsequent DBPCFC	9 patients (from 20)	
Total of DBPCFC	36 challenge tests	
Positive DBPCFC tests	16 positive reactions	
Negative DBPCFC tests	20 negative reactions	
	Positive DBPCFC reactions	Negative DBPCFC reactions
Significant ECP release	12/16	20/20
Negative ECP release	4/16	0/20

ECP = Eosinophilic cationic protein; DBPCFC = double-blind, placebo-controlled oral food challenge.

Discussion

GA as part of the food hypersensitivity complex poses often special diagnostic problems for immunologists as well as gastroenterologists, when the allergic reaction to foodstuffs or antigens is primarily confined to the GI tract [1–5]. In this case food hypersensitivity may lack a clear history of food adverse reactions as well as of characteristic extraintestinal features commonly seen in other forms of FA. In addition, prick skin test and (food-specific) IgE antibody determinations may fail to show a clear food-specific sensitization [3, 4, 6].

The clinical manifestations of GA are primarily offered by intestinal symptoms like chronic or intermittent diarrhea, vomiting, malnutrition, intestinal water and electrolyte secretion, intestinal blood and protein loss, abdominal pain, colicky symptoms or bloating. Since multiple (gastrointestinal) diseases may cause similar clinical pictures (infections, celiac disease, lymphoma, enzyme or primary immunodeficiencies, irritable bowel syndrome, inflammatory bowel disease, etc.) an exhaustive graduated diagnostic approach is often necessary for an exact identification of the underlying disorder (e.g. serology, stool cultures, H₂ breath tests, stool weight and fat determinations, exocrine pancreas function tests, small bowel biopsy, radiology and endoscopy with histology of small or large bowel, etc.) [2–6].

Because of an extremely broad spectrum of potential differential diagnosis [1–6], many patients with suspected GA have to undergo endoscopic examina-

tion to evaluate other methods. The primary aim of these investigations was to set up reliable methods for a quantitative measurement of ECP and T from gastrointestinal tissue, as well as to look for appropriate parameters in gut mucosa (mediator diagnostics) which might enable an early identification of or screening for patients with GA, already on the occasion of diagnostic colonoscopy. Therefore, the present study reports on the characteristics of ECP and mast cell T from human gut mucosa in 20 patients with manifest GA, which was each clinically confirmed after an extensive diagnostic approach by DBPCFC.

Although eosinophils or IgE-bearing plasma cells and mast cell infiltrates of the intestine have been observed for many years by morphological and immunofluorescence studies [1, 14–16], data about the actual tissue concentrations of their mediators are scarce. As expected, patients with AE were found to contain significantly higher mean tissue ECP and T levels in the lower GI tract than controls (table 1). These findings underline previous results about tissue histamine concentrations in patients with GA and indicate an intense accumulation of mast cells and eosinophils even in the hindgut of patients with manifest GA. However, more strikingly, these investigations revealed also that the gut tissue mediator levels decline markedly when patients were successfully treated by an effective allergen elimination diet (ECP > T). These results suggest that elevated ECP and T levels in patients with manifest abdominal symptoms due to GA may contribute to several pathophysiological events observed in intestinal allergic disorders. Therefore, it should be mentioned that ECP is well known to cause several toxic or noxious effects on human epithelial, smooth muscle or nerve cells, to enhance the mediator release from mast cells (e.g. histamine) and to interfere with blood coagulation, antibody production and T-cell function [17–20]. T has been found to induce proteolytic tissue damage and to mediate activation of complement (C_{3a} generation), fibrinogenolysis and collagenase [18, 21, 22]. In this respect it is likely that these strong mediator effects of ECP and T are responsible, at least in part, for several symptoms of manifest GA as well as for several pathophysiologic findings of acute GA like increased permeability indices, increased rates of antigen entry of human gut mucosa, enhanced water and electrolyte secretion or intestinal blood and protein loss [2, 4–6]. In addition, there is some evidence that ECP may influence (decrease) the production of IgA and alter T-cell functions (including oral tolerance) [17].

The differentiation of the tissue mediator levels within individual segments of the lower GI tract shows that ECP and T are physiologically present in all parts of gut mucosa, following normally a gradient of decreasing levels from the terminal ileum to the distal colon and rectum (ECP more pronounced than T). In contrast, patients with GA were found to possess not only increased, or significantly higher, mediator levels in particular segments of the gut, but also to exhibit an abnormal distribution of ECP and T. These mediators failed, in general,

segments (fig. 1, 2). This finding demonstrates on the one hand that eosinophils and mast cells may involve potentially every part of the lower GI tract in GA, and on the other hand that they are distributed rather in a patchy way than in a more regular one (ECP more pronounced than T), sometimes lacking within one patient equally raised tissue levels in all parts of the gut. This abnormal and more patchy distribution pattern may explain several clinically distinctive and local forms of GA like allergic proctitis, allergic colitis or gastritis syndrome and protein-losing enteropathy from small bowel mucosa [2, 14, 15]. It is also in accordance with the occurrence of local infiltrates of eosinophils in terminal ileum or other parts of the GI tract (e.g. esophagus) as described in food-hypersensitive patients [4]. In fact, among the studied patients with confirmed GA there were several individuals with high or excessive mediator levels in only one or two particular segments of the gut (e.g. terminal ileum and colon ascendens), while other parts had normal or slightly raised mediator levels. This may also explain sometimes the relatively high variations of the tissue mediator results found in GA. Moreover, in some of these patients with localized eosinophil or mast cell infiltrates a positive food challenge reaction could only be defined as 'immunologically mediated' when an intestinal lavage of the involved segment detected the presence of local intestinal food-specific IgE [13].

The proper incubation of gut mucosal samples by mucosa oxygenation revealed that both control patients as well as food-hypersensitive patients contain functionally reactive eosinophils and mast cells within human gut mucosa. This represents the physiological presence of vigorous immune effector cells in human gut tissue to protect the host against bacteria, fungi or parasites [5, 22]. However, in sensitized patients or, when having become in an activated state, eosinophils and mast cells are supposed to reflect the main pathophysiologic players in acute GA (apart from immunoregulating cells). This is also indicated by the fact that mononuclear or polymorphonuclear cell infiltrates are no characteristic histologic feature of GA, in contrast to inflammatory bowel disease, where several immune cell types appear to be involved in tissue inflammation [5, 16]. The vigorously enhanced spontaneous and anti-IgE-stimulated ECP release as well as the slightly to moderately increased T secretion in AE may further underline the dominant pathophysiologic role of eosinophils and mast cells in manifest GA. Furthermore, the release experiments with intact gastrointestinal tissue point out that the functional activity of eosinophils exceeds that of mucosal mast cells, which suggests that the eosinophil may be yet more dominantly and consistently or chronically involved in manifest GA than mast cells.

As shown previously with histamine release experiments, mucosa oxygenation enables the use of tiny endoscopic samples for the identification of mast cell- (and eosinophil) activating agents (e.g. antigens, drugs, bacteria, etc.) di-

rectly in the involved tissue sites in different disease stages [23–25]. Since recent findings have provided strong evidence for the important role of microenvironmental factors in regulating eosinophil and mast cell function [22], functional release experiments with food antigens were made with intact tissue samples (mucosa oxygenation). However, the allergen-induced ECP and T release did not evoke such an extent as obtained by anti-IgE (which might be related to methodical aspects like optimal allergen dilution). But allergens eliciting a positive DBPCFC reaction were each found to cause a significantly enhanced ECP or T kinetics, at least for the later time points of 120 and 240 min, than DBPCFC-negative antigens (fig. 3, 4).

This approach prompted us to prospectively compare the results of the intestinal mediator release with the outcome of DBPCFCs. As shown in table 2, these preliminary data from 9 patients indicate that the antigen-induced ECP release from gut mucosa is a useful, perhaps a diagnostic parameter to (pre)select or exclude antigens for DBPCFC, which should yet be performed as clinical confirmation of an underlying GA. A clinically relevant (causative) mediator release was defined as an antigen-induced ECP release which exceeds the double of the spontaneous release from the same patient. Under these conditions the mucosa oxygenation test with ECP achieved a sensitivity and specificity of 75 and 100%, respectively (table 2), when patients with manifest clinical symptoms due to GA were investigated for their provocative allergen(s). The sensitivity of this test might be further increased when more exact data are available about the appropriate (individual) site of biopsy taking, about the (individual) optimum allergen concentration, about the role of (individual) allergen-specific IgA binding or of unspecific tissue factors (mucin, bacterial or enzyme proteolysis) during the whole incubation time. The excellent specificity of the mucosa oxygenation test by ECP is striking and exceeds clearly that of histamine release experiments [23, 24], which may be explained on the one hand by the fact that ECP is a unique marker for eosinophil activation [18, 19], and on the other hand by the fact that histamine is a more delicate mediator than ECP. Histamine may be easily released by any sort of tissue damage (possibly biopsy taking) and its quantitative measurement using intact tissue samples may be influenced by the great activity of gut histamine catabolism [7, 9, 11]. In addition, histamine in intestine is not only stored in mast cells, but also in several other cell types (tissue-invading basophils, enterochromaffin cells, intraepithelial lymphocytes) and it may also be produced from bacteria or come from foodstuffs still contaminating the tissue in spite of having taken purgatives for colonoscopy [16, 23, 26, 27].

In summary, these investigations demonstrate that intestinal mediator diagnostics and mucosa oxygenation of intact tissue samples derived from the GI tract enable a clinically useful analysis of the gut tissue mediator content, including the study of their secretion pattern in response to different stimuli or drugs.

Such studies are not only useful, perhaps diagnostic, in GA, but they may also be warranted in several other disease states where increased mast cell or eosinophil numbers have been observed [5, 9, 16, 23, 28]. Elevated tissue ECP and T levels in combination with increased rates of spontaneous as well as anti-IgE or allergen-induced mediator release have been proven as highly suspicious of an underlying intestinal allergic disorder (e.g. GA) even in individuals with uncharacteristic anamnestic, clinical or laboratory findings.

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