Short communication

Airway mast-cell activation in asthmatics is associated with selective sputum eosinophilia

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Key words: asthma; eosinophils; neutrophils; sputum; tryptase.

Background: Tryptase is a serine endoprotease selectively released from mast cells. Although mast cells are known to be activated after experimental allergic provocation, their role in naturally occurring asthma is still debated.

Methods: We have investigated the levels of tryptase in the whole induced sputum collected from 51 asthmatics (31 atopic and 20 intrinsic) seen in our outpatient clinic and 22 normal nonatopic healthy volunteers. Tryptase was measured by a new immunoassay based on B12 monoclonal antibody recognition of total tryptase (UniCAP System, Pharmacia) with a sensitivity of 1 ng/ml.

Results: While being below the threshold of detection in all normal volunteers, tryptase was detectable in the sputum from 9/51 asthmatics (18%) including five atopic and four intrinsic asthma cases. In these patients, among whom three were asymptomatic asthmatics, the values ranged between 1 and 6.1 ng/ml. The asthmatics with detectable sputum tryptase had greater sputum eosinophil counts ($P<0.05$) but lower neutrophil counts ($P<0.05$) than those in whom tryptase was undetectable. When compared to control subjects, asthmatics without tryptase had still greater eosinophil counts ($P<0.0001$) but also raised neutrophil counts ($P<0.05$). No significant difference could be found between asthmatics with tryptase and those without tryptase with respect to the age, the baseline lung function, the methacholine bronchial responsiveness, and the frequency of treatment with inhaled steroids.

Conclusions: With the UniCAP System, tryptase was detectable in the sputum from 18% of asthmatics irrespective of atopy and current symptoms. Asthmatics with tryptase appeared to have a selective increase in sputum eosinophil counts while those without tryptase displayed a mixed sputum granulocyte infiltration with raised eosinophil and neutrophil counts.

Because it generates a large array of potent mediators after an IgE-mediated reaction, the mast cell has long been viewed as a pivotal cell in the pathophysiology of atopic asthma (1). Among the mediators secreted by mast cells is the 134-kDa serine endoprotease tryptase. This protease accounts for up to 20% of mast-cell protein content (2). In contrast to histamine, which is released from mast cells but also from basophils, tryptase is synthesized in negligible quantity in basophils and therefore appears to be a selective marker of mast-cell activation. Tryptase levels in BAL (3–5) and sputum (6, 7) were shown to be increased in asthmatics when compared to control subjects. If mucosal mast-cell degranulation is a well-documented event after a local allergenic provocation in experimental conditions (3, 8), it remains largely unknown whether airway mast-cell activation occurring under natural circumstances is associated with any particular asthma phenotype.

The purpose of our study was to assess the degree of airway mast-cell activation by measuring sputum tryptase levels in a large series of both atopic and intrinsic asthmatics recruited from our outpatient clinic. We also sought to establish whether the extent of mast-cell activation was associated with any particular features of the sputum cellular composition. Sputum levels of tryptase were determined in this study with a new immunoassay recently validated for serum measurements which allows detection of total tryptase (9, 10).

Material and methods

Subject characteristics

Fifty-one asthmatic subjects (mean age [range]: 45 years [15–75], male/female: 22/29) and 22 healthy nonatopic volunteers (mean age: 33 years [23–52], male/female: 13/9) participated in the study. Asthmatics were recruited from our outpatient clinic. Asthma was diagnosed on the basis of a clinical history of recurrent wheeze, breathlessness, or cough associated with bronchial hyperresponsiveness to methacholine ($PC_{20M} < 16$ mg/ml) when baseline FEV_1 was $\geq 70\%$ predicted or a significant reversibility of FEV_1 ($\geq 15\%$) when the baseline FEV_1 was $<70\%$ predicted. Methacholine bronchial responsiveness was measured according to a previously described protocol (11) with slight changes in the concentrations used. The first concentration to be inhaled was 0.06 mg/ml (instead of 0.03 mg/ml), and the challenge was carried out by successive inhalations of fourfold increased concentrations (instead of twofold) until a 20% fall in FEV_1 had occurred or a maximal methacholine concentration of 16 mg/ml had been inhaled. Mean (range) baseline FEV_1 was 86% predicted (32–115) in asthmatics and 104% predicted (85–127) in control subjects. Geometric mean (range) PC_{20M} was 1.5 mg/ml (0.04–16) in asthmatics and >16 mg/ml in healthy subjects. Thirty asthmatics were diagnosed as atopic on the basis of a positive skin prick test (wheal of >3 mm when compared to negative control) to at least one of the following common aeroallergens: house-dust mites; cat and dog dander; grass, tree, and weed pollens; molds; and feathers. Eleven of the 51 asthmatics were treated with inhaled steroids. All the subjects gave their written informed consent after the protocol had been approved by the hospital ethics committee.

Sputum induction

The subjects were premedicated with 400 μg inhaled salbutamol. Hypertonic saline (4.5%) was aerosolized by ultrasonic nebulizer Ultra Ned 2000 (De Vilbiss), with output set at 1.5 ml/min. The subjects wore a nose clip and quietly inhaled aerosol through a mouthpiece for up to four 5-min periods. After each inhalation, the subjects rinsed their mouths with water and dried them with tissue paper to minimize contamination with saliva. Then they coughed up sputum into a plastic container, which was immediately placed on ice until processing. Peak expiratory flow rate was measured after each 5-min inhalation period (Mini-Wright), and if it was greater than 250 l/min, the challenge was carried on. After challenge, the subjects were supervised for at least 1 h, and PEF was monitored regularly.

Sputum processing

The whole sputum was transferred into 50-ml polypropylene tubes (Becton Dickinson, Abingdon, UK) and weighed, and an equal weight of 0.01 M dithioerythritol (DTE; Fluka, Gillingham, Dorset, UK) solution added as a mucolytic. This was vortexed for 10 s, rocked for 30 min at room temperature, and again vortexed for 10 s. The samples were then filtered through a 70-μm strainer (Becton Dickinson), and the collected fluid was centrifuged at 400 *g* for 10 min at 4°C. The supernatants were removed and stored at −20°C. The cell pellets were resuspended in 1 ml of PBS without Ca^{2+} and Mg^{2+}, and cells were counted with a manual hemocytometer. The differential cell count was performed on cytospins stained with Diff Quick® after counting 600 cells (excluding squamous).

Tryptase assay

Tryptase was measured by a new commercially available immunoassay, the monoclonal antibody of which recognizes both the active β-tryptase and the inactive α-protryptase (B12 monoclonal antibody, UniCAP System, Pharmacia). To ensure adequate recovery of tryptase in the sputum samples, we performed spiking experiments by adding known concentrations of tryptase in a pool of sputum samples without tryptase. The added concentrations were 2.5, 6.5, 25, and 100 ng/ml, and the recovered concentrations were 2.3, 6, 22, and 105 ng/ml, indicating a recovery close to 100%.

Statistical analysis

Cell counts and mediator levels were expressed as median (range) and compared between asthmatics and healthy subjects by nonparametric tests. Comparison between two groups was performed with the Mann–Whitney test. When three groups were compared together, we used the Kruskal-Wallis test and, in case of significance, the Mann–Whitney test to perform pairwise comparisons. Comparisons of age, FEV_1, and PC_{20M} between asthmatics with tryptase and those without tryptase were performed by the unpaired Student's *t*-test. Comparisons of qualitative parameters such as atopy, smoking habits, and treatment by inhaled steroids between tryptase-positive and tryptase-negative asthmatics were performed by Fisher's exact test.

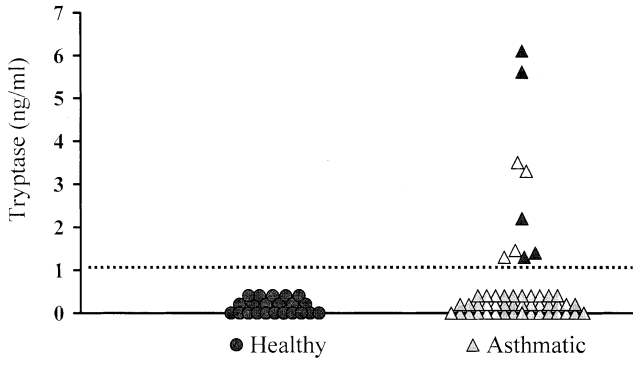

Figure 1. Sputum tryptase levels in healthy subjects (circles) and asthmatics (triangles). Detectable tryptase from atopic asthmatics is indicated by solid triangles and that from intrinsic asthmatics by open triangles. Dashed line represents sensitivity of immunoassay.

Results

When compared to healthy subjects, asthmatics had a greater proportion of eosinophils ($P<0.0001$) and neutrophils ($P<0.05$) in their sputum but a lower proportion of macrophages ($P<0.001$). When results were expressed as absolute values, asthmatics were still distinguished from healthy subjects by increased eosinophil counts ($P<0.0001$) (Table 1).

Tryptase was undetectable in all healthy subjects and detectable in the sputum from only nine of 51 asthmatics (Fig. 1). In these subjects, the values ranged from 1 to 6.1 ng/ml. The subjects who had detectable levels of tryptase included five atopic asthmatics and four intrinsic asthmatics. Among the nine patients in whom tryptase was found to be detectable, three had to be considered intermittent atopic asthmatics as they reported symptoms less than once per week. When compared to asthmatics without detectable tryptase, those with detectable tryptase in their sputum had a greater relative sputum eosinophil count (9.2% [2.6–74] *vs* 2.4% [0.2–90], $P<0.05$) but a lower relative sputum neutrophil count (16.2% [2.2–59] *vs* 44.2% [0–94], $P<0.05$) (Fig. 2). When cell counts were expressed as absolute values, this observation held for eosinophils ($P<0.05$), but no longer for neutrophils ($P=0.2$). In asthmatics, there was a significant positive correlation between sputum tryptase levels and the sputum eosinophil count however the eosinophil count was expressed ($r_s=0.41$, $P<0.001$ when expressed as a percentage and $r_s=0.31$,

Table 1. Sputum total and differential cell counts in healthy and asthmatic subjects

	Healthy subjects ($n=22$)	Asthmatics ($n=51$)
% Squamous cells	8.5 (0–29)	9 (2–29)
Total nonsquamous cells $\times 10^6$/g	0.9 (0.06–3.4)	1.22 (0.06–8.9)
% Macrophages	56.4 (16.8–93)	33.4 (0–72)**
Macrophages $\times 10^3$/g	517 (55–2238)	402 (0–2328)
% Lymphocytes	1.5 (0–5.2)	0.8 (0–12)
Lymphocytes $\times 10^3$/g	12 (0–150)	7 (0–331)
% Neutrophils	20.2 (0.8–81.4)	35.8 (0–99.4)*
Neutrophils $\times 10^3$/g	226 (0–2751)	310 (0–8837)
% Eosinophils	0 (0–2.3)	3.2 (0.2–90)****
Eosinophils $\times 10^3$/g	0 (0–34)	31 (1–4851)****
% Epithelial cells	9.8 (0.6–33.4)	6.4 (0–46.3)
Epithelial cells $\times 10^3$/g	79 (1–431)	82 (1–581)

Results are expressed as median (range).
*Indicates significance *vs* healthy; *$P<0.05$, **$P<0.01$, and ****$P<0.0001$ (Mann–Whitney test).

$P<0.05$ when expressed as absolute value). In the same group, sputum tryptase levels inversely correlated with the sputum neutrophil count expressed as a percentage ($r_s=-0.23$, $P=0.05$), but not with neutrophils expressed as absolute value ($r_s=-0.17$, $P>0.05$). There was no difference between the tryptase-positive and the tryptase-negative asthmatics regarding the other cell types. When compared to control subjects, asthmatics without tryptase still had a clear increase in sputum eosinophil counts ($P<0.0001$) but also a raised sputum neutrophil count ($P<0.05$) (Fig. 2). This was still valid for the cell counts expressed in absolute values ($P<0.0001$ for eosinophils and $P=0.06$ for neutrophils, respectively).

There was no significant difference between the asthmatics with and those without tryptase in their sputum with respect to age (mean ± SD: 38 ± 13.5 *vs* 45 ± 17 years), FEV_1 (mean ± SD: 87 ± 22 *vs* 86 ± 19 years), PC_{20M} (geometric mean [range] 0.8 [0.05–14] *vs* 1.8 [0.04–16]), and the frequency of treatment with inhaled steroids (1/9 *vs* 10/42) ($P>0.05$ for all variables).

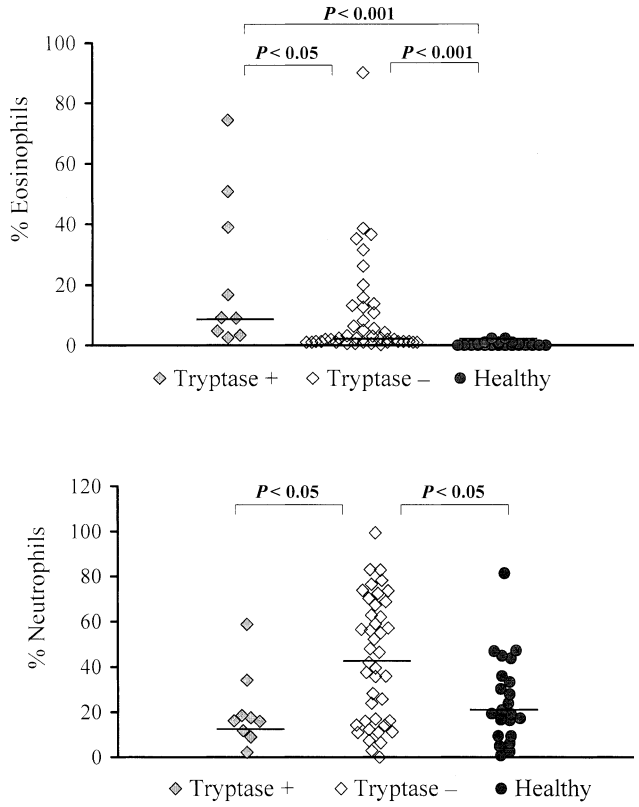

Figure 2. Sputum eosinophil (upper panel) and neutrophil counts (lower panel) according to presence or absence of tryptase in fluid phase of sputum samples. Horizontal bars represent median value.

Discussion

Our study shows that tryptase was detectable in the sputum of 18% of asthmatics, but this protease was virtually undetectable in healthy volunteers. The presence of tryptase in the sputum was found indiscriminately in atopic and intrinsic asthmatics. Detectable levels of tryptase in asthmatics were associated with prominent airway eosinophilia without increased neutrophilia.

In this study, we found a relatively low percentage of asthmatics with detectable levels of tryptase in their sputum. This contrasts with some previous work, including ours, reporting that tryptase was detectable in the whole sputum of a majority of asthmatics (7, 12). Discrepancies between previous reports and our present study might be related to the type of immunoassay employed. Tryptase exists in two distinctive forms, the active β-tryptase secreted during mast-cell degranulation and the α-protryptase constitutively released (2). In this study, we have used for the first time a new immunoassay recognizing both β-tryptase and α-protryptase (monoclonal antibody B12, UniCAP System, Pharmacia), and therefore measuring total tryptase, while the immunoassay used in previous studies recognized only β-tryptase (G5 monoclonal antibody, RIACT, Pharmacia). This should have enhanced the sensitivity of the assay, as has been demonstrated for serum measurements. Indeed, the use of the B12 monoclonal antibody allows the detection of tryptase in the serum of normal subjects, unlike the G5 monoclonal antibody (9).

However, our data indicate that the increased sensitivity found for serum measurements does not necessarily apply to measurement in other biological fluids such as sputum. This suggests that the inactive α-protryptase is not an important component of the pool of tryptase in the airways, as it is in the serum. While the sensitivity of the new immunoassay based on the B12 monoclonal antibody is 1 ng/ml, the sensitivity of that based on G5 monoclonal antibody (RIACT) was found to be 0.5 ng/ml. If this relative loss in sensitivity for the β-tryptase may partly explain the lower rate of tryptase-positive asthmatics in this study, it is certainly not the major reason, as the majority of asthmatics tested in previous studies with the RIACT were found to have tryptase levels higher than 1 ng/ml (5, 12).

On the other hand, we do not believe that the use of DTE as a mucolytic has adversely affected the immunoassay, as spiking experiments in this study clearly show that treating the samples with DTE allows a full recovery of tryptase. In addition, tryptase levels in samples treated with DTE have been found to be greater than in those treated with PBS (13). Certainly, the best approach to analyze any difference

between the two immunoassays would have been to compare their sensitivities in the same study, but, unfortunately, the RIACT was no longer available by the time we carried out this study. Instead, to establish the lack of sensitivity of the immunoassay, we might also consider real differences in the features of the airway inflammatory process between asthmatics in this study and those previously investigated. As local release of tryptase is well documented after experimental mucosal allergen exposure (3, 8), we can hypothesize that differences in the population studied reflect varying degrees of natural allergen exposure. It is worth noting, however, that tryptase could also be detected in some of our asthmatics in whom no sensitization to common aeroallergens was found by skin prick tests. This emphasizes the similarities in the airway inflammatory process found in atopic and ''intrinsic'' asthma (14), and it also suggests that mast-cell activation may occur under other circumstances than sole allergen exposure.

The interesting point of this study is the fact that asthmatics with detectable levels of tryptase in their sputum also had a greater eosinophil count but a lower neutrophil count than those without tryptase. This association between sputum tryptase levels and sputum eosinophil counts is in line with reports showing a positive correlation between mast-cell and eosinophil numbers infiltrating the nasal epithelium in asthmatics sensitized to grass pollen (15) as well as between BAL cell lysate histamine and BAL eosinophil numbers in a heterogeneous group of asthmatics (16). Taken together, these observations suggest a functional relationship between airway mast-cell number and/or activation and airway eosinophil recruitment and/or subsistence. Recent *in vitro* findings lend support to this hypothetic relationship. Tryptase, a product of mast-cell degranulation, has recently been shown to be able to stimulate eosinophil chemotaxis (17), and IL-5, which can be released from mast cells after an IgE-mediated stimulation (18), is a potent factor enhancing eosinophil chemotaxis and survival (19).

On the other hand, the asthmatics with tryptase in their sputum appeared to have a relatively low proportion of neutrophils infiltrating their airways, an observation which indicates that naturally occurring airway mast-cell activation is accompanied by selective eosinophilic inflammation. The reason for this selective eosinophil recruitment is unclear, but it might be related to the concomitant release of mediators, such as histamine, that can inhibit neutrophil chemotaxis (20). It is noticeable that asthmatics without tryptase had a mixed airway granulocytic infiltration with raised sputum eosinophil and neutrophil counts when compared to control subjects. Thus, neutrophils seem to migrate in the asthmatic airways as the role of mast cells in the airway inflammatory process becomes less important. This apparent antagonism between neutrophils and mast cells has been recently documented in the airways of severe asthmatics where eosinophils and neutrophils appear to be abundant with a poor contribution of mast cells to the inflammatory process (21). In our study, we could not find significant differences between tryptase-positive and tryptase-negative asthmatics in terms of age, baseline lung-function impairment, and bronchial methacholine hyperresponsiveness, but the study was not designed to look specifically at severe asthmatics. It is worth noting that among the nine asthmatics found to be tryptase positive, three were asymptomatic, a fact which stresses that the extent of mast-cell activation is poorly related to the clinical severity of asthma. This is in line with a previous study failing to show any relationship between the Aas score and BAL tryptase levels in asthma (5), as well as with our recent observation that sputum tryptase levels were not greater in severe than intermittent asthma (22).

We conclude that, with the new UniCAP System based on B12 monoclonal antibody, tryptase is detectable in approximately one-fifth of asthmatics while remaining undetectable in all healthy nonatopic subjects. In asthmatics, tryptase can be detected in the sputum irrespective of atopy or the presence of current symptoms. The presence of tryptase is associated with a selective sputum eosinophilia, while its absence coincides with a mixed granulocytic infiltrate.

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