

Original article

The extracellular deposition of mast cell products is increased in hypertrophic airways smooth muscles in allergic asthma but not in nonallergic asthma

Background: Bronchial asthma is characterized by airways smooth muscle hypertrophy and infiltration of mast cells in the bronchial mucosa. The aim of this investigation was to study the distribution of mast cells in different compartments in the bronchial mucosa of allergic and nonallergic asthma in relation to airways remodeling.

Methods: Bronchial biopsies were obtained from 29 subjects with allergic and nonallergic asthma and healthy controls. The biopsies were stained for mast cells by means of the tryptase specific antibody AA1. Extracellular deposition of mast cell products were judged on a semi-quantitative scale. Mast cells per mm² were counted in epithelium, lamina propria and the smooth muscle compartment. Smooth muscle was visualized by actin antibodies and the proportion of staining of the biopsy estimated. Laminin and tenascin layers were visualized by their respective antibodies.

Results: Airways smooth muscle thickness was greater in allergic vs nonallergic asthma ($P < 0.001$). Mast cells were increased in all three compartments in both allergic and nonallergic asthma, with significantly higher numbers in smooth muscles in allergic asthma ($P < 0.03$). The extracellular deposition of mast cell products was more common in allergic than nonallergic asthma in lamina propria and smooth muscles ($P = 0.025$; $P = 0.002$, respectively). In patients with allergic asthma the numbers of mast cells with extracellular deposition of mast cell products were significantly correlated to the thickness of the laminin and tenascin layers.

Conclusion: Our results suggest that there are large differences between allergic and nonallergic asthmatics as to mast cell activation and airways smooth muscle thickness. Our data implies that mast cells are causally involved in structural alterations in allergic asthma.

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Asthma is characterized by the accumulation of inflammatory cells in the submucosa, gland hyperplasia, thickening of subepithelial layers (1, 2) and hyperplasia of airway smooth muscle cells (3–6). Most of these structural changes may be induced by inflammatory cell products (1, 7, 8).

The mast cell appears to play an important role in the orchestration of inflammation in the airway. The mast cell is able to release potent inflammatory mediators, such as histamine, pro-inflammatory cytokines including IL-4, IL-5, and IL-13 (9–12) and proteases including tryptase and chymase, which potentially damage and activate the bronchial epithelium, connective tissue, and may contribute to airway wall remodeling (13). In a previous investigation, we found an abundance of mast cells in the bronchial mucosa of

patients with both allergic and nonallergic asthma and in allergic asthma the presence of mast cells was correlated to structural changes such as thickening of the basement membrane (1). Recently Brightling and co-workers reported that the accumulation of mast cells in the airways smooth muscle compartment was increased in asthmatics and that this increase was related to bronchial hyper-responsiveness (14). Taken together these data suggested that the mast cells might be actively involved in the remodeling of the airways in allergic asthma.

The aim of this investigation was to study the distribution of mast cells and signs of their activation in different compartments in the bronchial mucosa of patients with allergic and nonallergic asthma in relation to airways remodeling.

Material and method

Subjects

Bronchial biopsies were collected from 29 nonsmoking adults divided into the following groups: healthy controls ($n = 7$), patients with allergic asthma ($n = 13$) and patients with nonallergic asthma ($n = 9$) (Table 1). All patients had a clinical asthma diagnosis, current asthma symptoms and increased responsiveness to inhaled methacholine. The allergic asthma patients all had a positive skin prick test (≥ 3 mm) for at least one common allergen [birch, timothy grass (*Phleum pratense*), mugwort (*Artemisia vulgaris*), cat, dog, horse, house dust mite (*Dermatophagoides pteronyssinus*), *Cladosporium*, and *Alternaria*], while the nonallergic asthma patients and the controls all had a negative skin prick test. All patients with allergic asthma were examined outside the birch and grass pollen season (April to August).

All but one allergic and one nonallergic patient with asthma were on regular treatment with inhaled glucocorticosteroids (budesonide 200–800 $\mu\text{g/day}$) and inhaled β_2 -agonists as needed. The average use of inhaled glucocorticosteroids was similar in the two asthma groups. A more detailed description of the study population has been presented in a previous report (1).

Procedure

Fiber bronchoscopy was performed according to a previously described protocol (1).

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at the Faculty of Medicine at the University of Uppsala.

Immunohistochemistry

The mast cells were identified with monoclonal antibodies on frozen sections. The anti-tryptase antibody 1, AA1 (M 7052, Dakocytomation, Glostrup, Denmark), for the identification of mast cells, was used at a final concentration of 0.21 $\mu\text{g/ml}$. Monoclonal antibodies to anti-human smooth muscle actin (M 0851; Dakocytomation, Glostrup, Denmark) were used on frozen sections at concentration of (37.5 $\mu\text{g/ml}$).

The bronchial biopsy specimens taken from the upper lobe and were frozen immediately (1). The sections were thawed and then fixed with undiluted Ortho Permeafix (Ortho Diagnostics, Raritan, NJ, USA) for 40 min at room temperature prior to incubation with

antibodies to the mast cell marker tryptase. Prior to the incubation with antibodies to laminin, tenascin, anti-human smooth muscle actin the sections were fixed with acetone for 10 min at room temperature. After the appropriate fixation procedure the sections were incubated with monoclonal antibodies at room temperature in a humid chamber for 1 h and the biopsies were processed as previously described (1).

Microscopic evaluation of sections

The number of cells in the mucous membrane was counted in the whole specimen and the data were converted to an area of 1 mm^2 of tissue. Cell numbers were counted separately in three different compartments, i.e. in epithelium, in lamina propria and in smooth muscle. The areas of the different compartments were outlined and the contained area was measured using image analysis with the Leica DMLB microscope (Wetzlar GmbH, Germany) equipped with a Leica Microsystems digital camera (DC 300F) connected to a computer. The images were captured and saved in the computer for further evaluation using the software package Qwin v2.7 after calibration with the aid of a stage squared micrometer. In each biopsy two subsequent sections were evaluated. The variation in cell counts and estimation of structural changes between the two microscopic sections varied between 3 and 5% (% coefficient of variation).

The extracellular deposition of mast cell products and nonextracellular deposition of mast cell products were identified in the lamina propria and in smooth muscles. Mast cells were categorized as mast cells with extracellular deposition of mast cell products when extracellular deposition of anti-tryptase stained material was observed.

The epithelial integrity of the bronchial wall of the subjects from the different groups was assessed by light microscopy in 4 μm thick frozen sections stained with Mayer's hematoxyline. Epithelial integrity was estimated using a $\times 10$ objective and sidearm light microscopic attachment that allowed the image of the section to be projected onto a computer screen. The total length of the basement membrane and the length of intact epithelium in the section through each biopsy were determined. The total length of intact epithelium was expressed as a percentage of the total length of basement membrane present in each biopsy. The area of smooth muscle (mm^2) was identified by positive staining for actin. This area was divided by the whole area of the biopsy (mm^2) and the amount of smooth muscle was expressed as the proportion (%) of tissue in the biopsy.

The measurement of the thickness of the tenascin and laminin layers (in μm) was performed in 4- μm thick immunolabeled frozen sections using a $\times 40$ objective and a computerized image analysis system as described above.

All specimens were coded and examined by the microscopist without any knowledge of the diagnoses of the patients.

Statistical analysis

All statistics were calculated using nonparametric tests. Comparisons between the three groups, healthy subjects, allergic and nonallergic asthmatics were initially performed by means of analysis of variance (Kruskal–Wallis test). In case of significance paired group comparisons were performed with the Mann–Whitney U -test. For correlations within groups, Spearman's rank correlation test was used. All calculations were performed using the statistical package Statistica for Windows v. 6.0 from Statsoft (Tulsa, OK, USA). A P -value of < 0.05 was regarded as statistically significant.

Table 1. Patient characteristics (median or range)

	Healthy control ($n = 7$)	Allergic asthma ($n = 11$)	Nonallergic asthma ($n = 9$)
Age (years)	25 (22–43)	37 (19–63)	41 (17–62)
Sex (M/F)	2/5	2/9	2/7
FEV ₁ (% pred)	98 (71–120)	100 (72–132)	86 (72–97)
FVC (% pred)	98 (78–109)	102 (86–141)	87 (76–96)
Symptom score	0 (0–1)	2 (0–4)	2 (1–2)
PEF-variability (%)	5 (3–9)	10 (6–22)	10 (5–20)
PC ₂₀ (mg/ml)	–	3.4 (0.07–32)	8.7 (1.0–32)
Pollen allergy	0	10/13	0
Pet allergy	0	13/13	0
Mite allergy	0	5/13	0
Mould allergy	0	3/13	0

Results

There were no statistically significant differences between allergic and nonallergic patients with asthma in terms of lung function, symptom score, peak flow variability or PC₂₀ (Table 1). All the patients with allergic asthma were sensitized to pet allergens and most of them also to pollen.

Thickness of smooth muscle in asthma patients

The thickness of airway smooth muscle in patients with allergic asthma was increased compared with nonallergic asthma and the controls ($P < 0.001$), whereas the thickness was similar in nonallergic asthma and controls (Fig. 1).

Mast cells in different compartments in asthma patients

Both allergic and nonallergic patients with asthma had a higher number of mast cells (AA1-positive cells) in epithe-

lium, lamina propria and the airway smooth muscle compared with the controls. The numbers of mast cells in smooth muscles were higher in allergic asthma as compared with nonallergic asthma ($P = 0.03$). No significant differences were found in the two other compartments (Fig. 2).

Extracellular deposition of mast cell products in asthma patients

Examples of mast cells with and without extracellular deposition of mast cell products in lamina propria and smooth muscle are shown in a biopsy from a patient with allergic asthma (Fig. 3A and B). Signs of secretion of tryptase were present and often quite extensive in biopsies from the allergic asthma patients as compared with the findings in the healthy control ($P < 0.001$). Extracellular deposition of mast cell products was also significantly higher in both compartments in patients with allergic asthma as compared with the nonallergic asthma group ($P = 0.025$ and $P = 0.002$) (Fig. 3C and D). In nonallergic asthma extracellular deposition of mast cell products were observed more often than among the

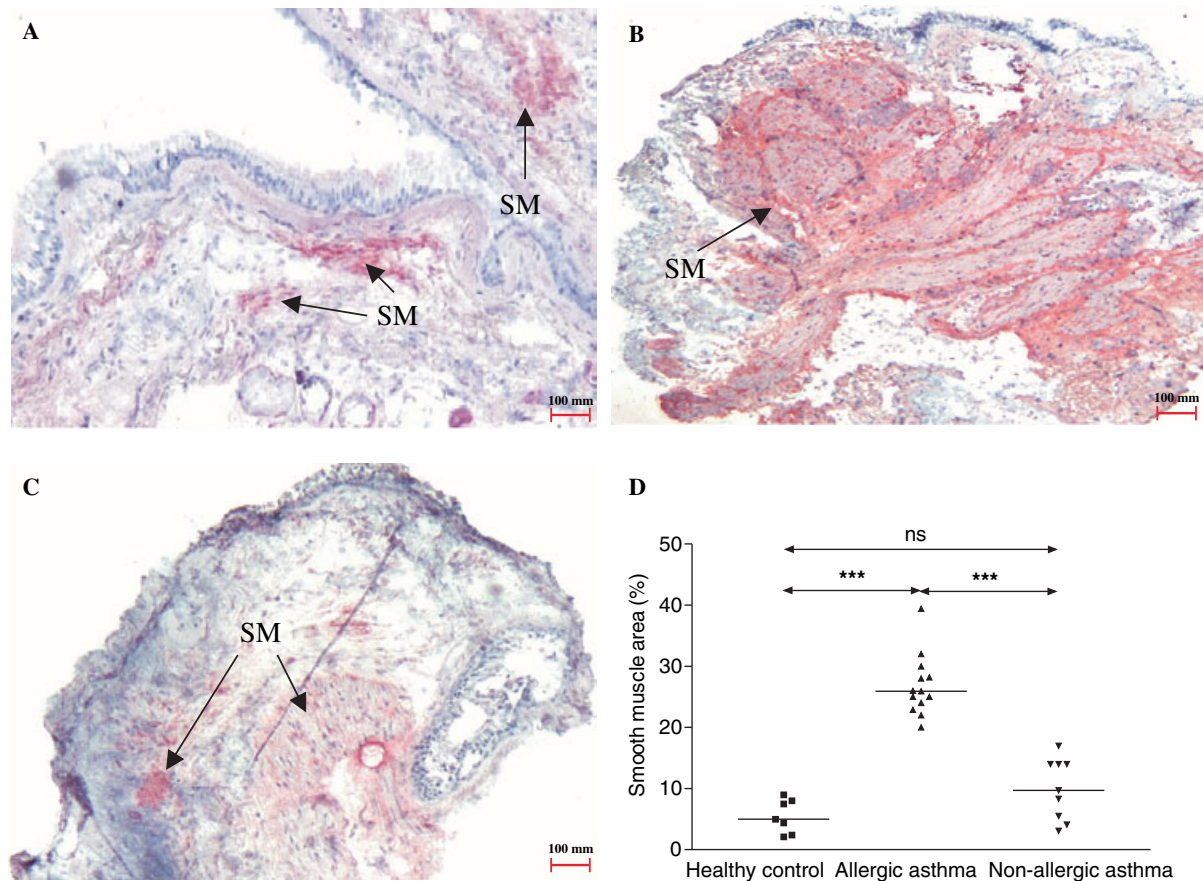


Figure 1. Cryostat sections of bronchial biopsies from healthy controls, patients with allergic asthma and nonallergic asthma. Light microscopic examination of the thickness of the airways smooth muscle (SM) in healthy control (A), allergic asthma (B), and nonallergic asthma (C). Smooth muscles are indicated by arrows. Original magnification ($\times 40$). The scale bars (100 μm) are shown in the figures. The comparison of airway smooth muscle area (%) in healthy controls, patients with allergic asthma and nonallergic asthma (D).

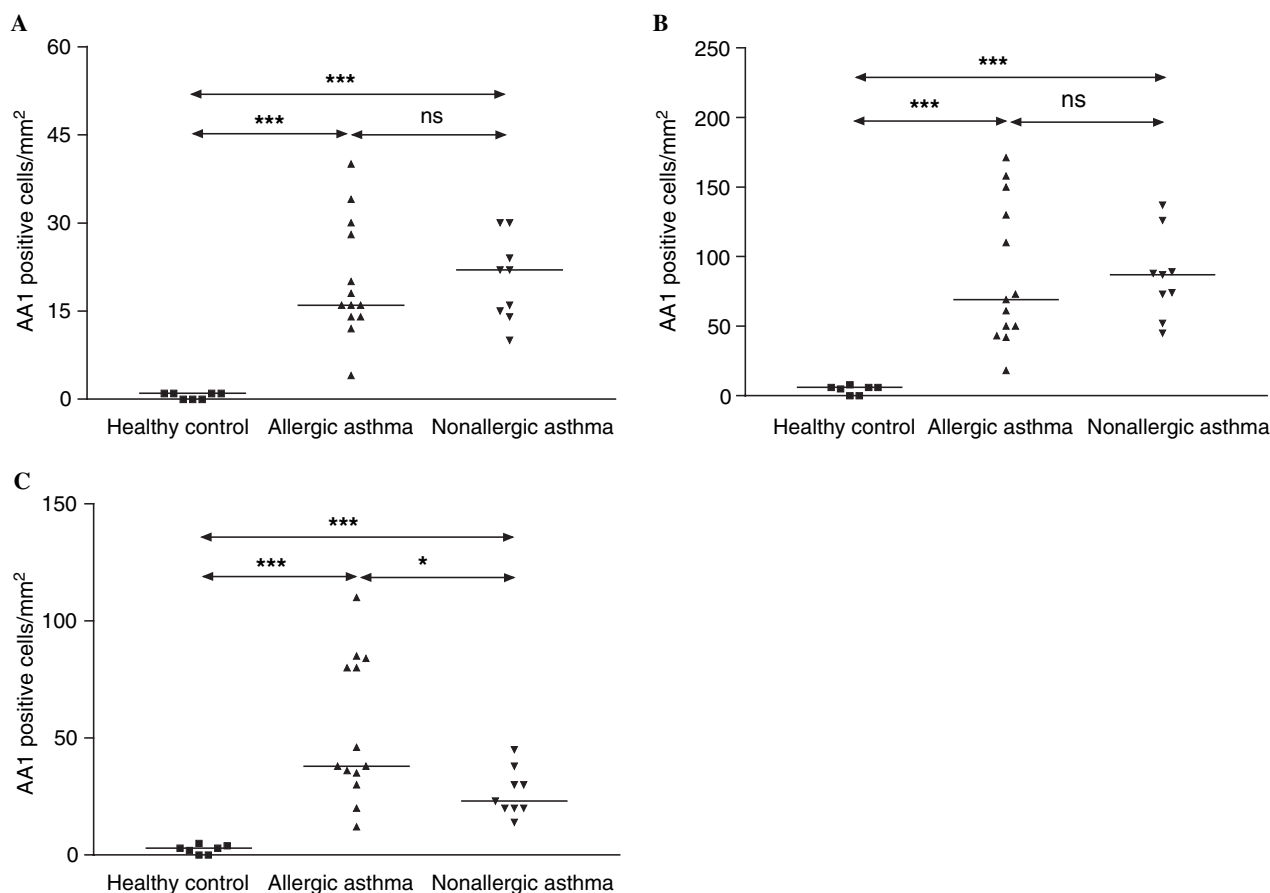


Figure 2. Comparison of the number of anti-tryptase antibody 1 (AA1) positive cells in biopsies of healthy controls, allergic asthma and nonallergic asthma. Epithelium (A), lamina propria (B) and smooth muscle (C).

controls ($P < 0.001$). The proportions of mast cells with extracellular deposition in lamina propria were 0% (0–12%) (median and range) in healthy controls, 31% (23–44%) in patients with allergic asthma and 17% (12–23%) in patients with nonallergic asthma ($P = 0.0001$, allergic vs nonallergic asthma). The proportions of mast cells with extracellular deposition in smooth muscles were 0% (0–25%) in healthy controls, 39% (25–46%) in allergic asthma and 22% (20–30%) in nonallergic asthma ($P = 0.0006$ allergic vs nonallergic asthma).

Correlations between mast cells and structural changes in the bronchial mucosa

In patients with allergic asthma the total numbers of mast cells and the numbers of mast cells with extracellular deposition in lamina propria and in smooth muscle were significantly correlated with the thickness of the tenascin and laminin layers (Table 2), whereas no such correlation was found to mast cells in epithelium. No correlations were found between mast cells and the thickness of smooth muscle or epithelial integrity.

In the groups of patients with nonallergic asthma and healthy controls no significant correlations were found

between the numbers of mast cells in any compartment and structural changes.

Discussion

The main findings of this study were differences in mast cells distributions in the bronchial mucosa of allergic and nonallergic asthma, with mast cells in patients with allergic asthma being accumulated in the smooth muscle compartment to a higher extent than in nonallergic asthma. Mast cells in allergic asthma also showed more general signs of activation with deposition of extracellular mast cells products, than in nonallergic asthma. Furthermore, in allergic asthma we found positive correlations between the number of mast cells in lamina propria and smooth muscles and the thickness of the tenascin and laminin layers, suggesting different roles of mast cells in allergic and nonallergic asthma. Our study also confirmed that patients with asthma have an increased thickness of airways smooth muscle, but that this thickening was only apparent in subjects with allergic asthma.

The findings of an increase in the airways smooth muscle in our asthmatic patients is not surprising, since

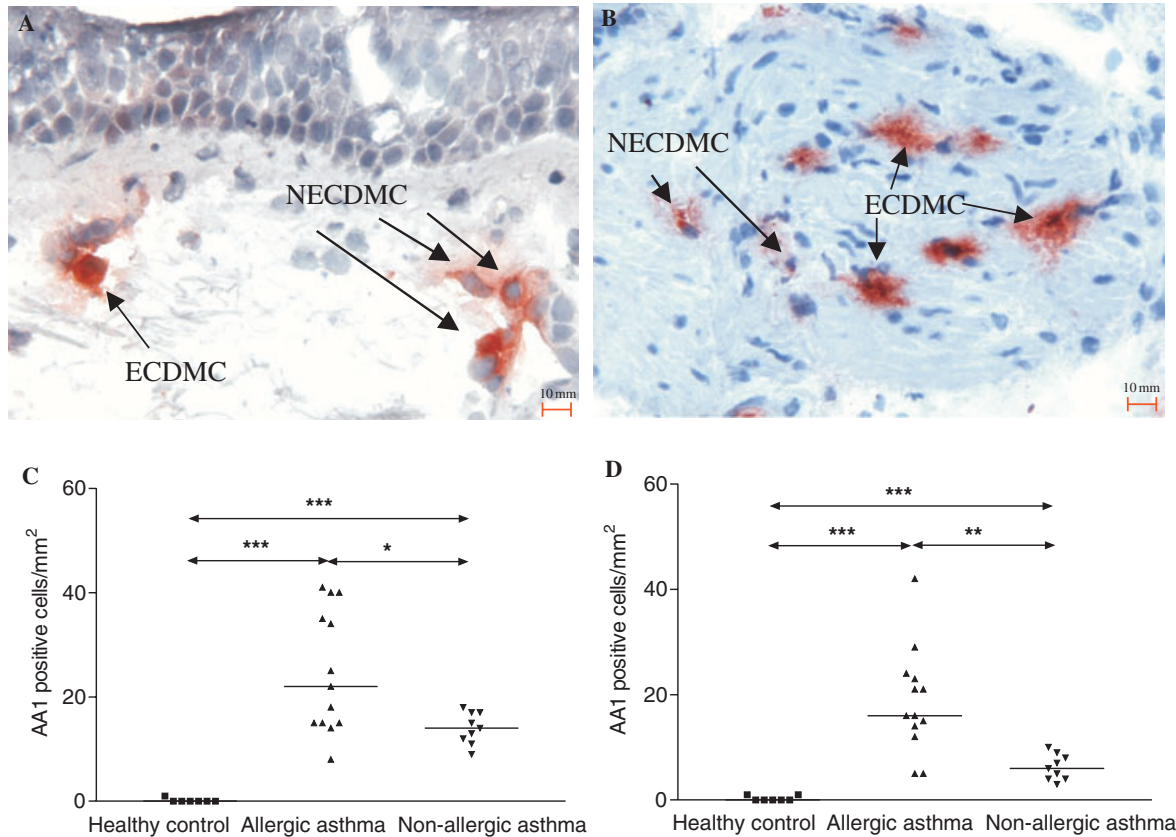


Figure 3. Bronchial biopsy specimens from a patient with allergic asthma stained for AA1 show mast cells with (ECDMC) and without (NECDMC) extracellular deposition of mast cell products and nonextracellular deposition of mast cell products in lamina propria (A) and smooth muscle (B). Mayer's hematoxyline. Original magnification: $\times 340$. The scale bars (10 μ m) are shown in the figures. The numbers of mast cells deposition of mast cell products in lamina propria (C) and smooth muscle (D).

Table 2. Correlation (*r*) between structural changes and mast cells with extracellular deposition in different compartments in patients with allergic asthma

	Thickness of laminin layer	Thickness of tenascin layer
Mast cells in whole biopsy	0.57*	0.62*
Mast cells in lamina propria	0.60*	0.62*
Mast cells with extracellular deposition in lamina propria	0.73**	0.69**
Mast cells in smooth muscle	0.59*	0.68*
Mast cells with extracellular deposition in smooth muscle	0.60*	0.70**

* $P < 0.05$, ** $P < 0.005$ (Spearman rank correlation).

this observation has been made repeatedly in many studies since the 1920s (3–6, 15–17). The more unexpected finding was the large difference in thickness that was observed between the two asthma groups. This finding is in line with our previous observation of clear differences in other structures of the bronchi between allergic and nonallergic asthma (1). Similar to a recent study, we found infiltration of mast cells in all compartments of the bronchi in both asthma groups (14) but the accumulation

of mast cells was more pronounced in the smooth muscle compartment in allergic than nonallergic asthma. We also found that mast cells in the bronchial mucosa of allergic asthmatics showed more signs of activation with extracellular deposition of tryptase than mast cells from nonallergic asthmatics. Signs of mast cell activation in allergic asthma have been indicated by others (14). However, in that study no differences between allergic and nonallergic asthmatics were observed (14), which may be due to the limited number of nonallergic asthmatics included in the study. The mechanisms that may activate mast cells in the bronchial mucosa in allergic asthma are likely related to specific allergens and their reaction with IgE on the mast cells especially since all the patients with allergic asthma were sensitized to perennial allergens such as pets (18).

In our previous study (1), we showed positive correlations between mast cell numbers in the bronchial mucosa of allergic asthmatics and the thickness of either the laminin or the tenascin layers. When evaluating the numbers of mast cells with extracellular deposition of mast cell products in lamina propria and smooth muscles in the present study the correlations to the thickness of

laminin and tenascin became somewhat stronger, indicating a relationship between the secretion of tryptase and other mediators and the production of these matrix proteins. Thus, our data support earlier conclusions (1) of a role of mast cells and mast cell products in the remodeling process of allergic asthma.

We did not find a correlation between smooth muscle thickening and mast cell numbers in the mucosa in allergic asthmatics. The lack of correlation does not exclude an involvement of mast cells in the process of smooth muscle growth as tryptase and other products secreted by mast cells have been shown to stimulate growth of smooth muscles *in vitro* (19). In addition to being an important stimulus for smooth muscle cell proliferation tryptase may also potentiate the contractile response to agonists such as histamine in asthmatic airways, by acting on proteinase-activated receptor-2 (8, 20). A potential role in tissue remodeling has been underscored by the finding that purified human lung tryptase can act as a potent mitogen for fibroblasts (21, 22), epithelial cells and dermal vascular endothelial cells (23). Tryptase can also degrade intact type VI collagen microfibrils (24). Tryptase released during mast cell degranulation could provide a stimulus both for fibroblast proliferation and collagen synthesis, resulting in increased collagen deposition in fibrotic lesions. Because human mast cells are the predominant cells that are activated during an allergen challenge, they may produce the cytokines that are responsible for the differentiation of smooth muscle cells to myofibroblasts,

which are then recruited and accumulated beneath the basement membrane (25).

The numbers of mast cells per mm² found in our biopsies are higher than those presented by others (14). There are probably several reasons for this. First of all sections prepared from frozen biopsy material regularly show larger numbers of mast cells than those found in plastic embedded material. This difference is probably because of the fact that the antigen is better accessible in cryosections, whereas in plastic sections the morphology and structure of cells are better preserved. Secondly, the fixation with Permeafix, that is used in our studies, seems to be a superior fixation agent for most cellular antigens (1).

In conclusion, our results suggest that there are large differences between allergic and nonallergic asthmatics as to mast cell activation and airways smooth muscle thickness. Our data implies that mast cells are causally involved in structural alterations in allergic asthma.

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