

MAST-CELL INFILTRATION OF AIRWAY SMOOTH MUSCLE IN ASTHMA

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ABSTRACT

Background Asthma and eosinophilic bronchitis are characterized by similar inflammatory infiltrates in the submucosa of the lower airway. However, eosinophilic bronchitis differs from asthma in that there is no variable airflow obstruction or airway hyperresponsiveness in the former condition. We tested the hypothesis that there were differences between the two conditions in the microlocalization of mast cells within the airway smooth muscle.

Methods Immunohistochemical analysis of bronchial-biopsy specimens was completed in 17 subjects with asthma, 13 subjects with eosinophilic bronchitis, and 11 normal controls recruited from two centers.

Results Both groups with disease had a similar degree of submucosal eosinophilia and thickening of the basement membrane and lamina reticularis. By contrast, the number of tryptase-positive mast cells in the bundles of airway smooth muscle from subjects with asthma (median, 5.1 mast cells per square millimeter of smooth muscle [range, 0 to 33.3]) was substantially higher than that in subjects with eosinophilic bronchitis (median, 0 mast cells per square millimeter; range, 0 to 4.8) and that in normal controls (median, 0 mast cells per square millimeter [range, 0 to 6.4]; $P < 0.001$ for the comparison among the three groups). T cells and eosinophils were not usually seen in the airway smooth muscle in any of the groups.

Conclusions The infiltration of airway smooth muscle by mast cells is associated with the disordered airway function found in asthma. (N Engl J Med 2002; 346:1699-705.)

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ASTHMA is characterized physiologically by variable airflow obstruction and airway hyperresponsiveness. Pathologically, asthma is characterized by the accumulation of eosinophils and CD4⁺ lymphocytes in the submucosa, mucous-gland hyperplasia, thickening of the subepithelial collagen layer, submucosal matrix deposition, mast-cell degranulation, and hypertrophy and hyperplasia of the airway smooth muscle.¹

The extent to which airway inflammation and airway hyperresponsiveness in patients with asthma are related to one another remains controversial.² However, there is a clear dissociation between airway inflammation and airway hyperresponsiveness in patients with eosinophilic bronchitis^{3,4} — a condition characterized by cough that is responsive to cortico-

steroids and eosinophilia detectable in sputum without variable airflow obstruction or airway hyperresponsiveness.⁵⁻⁷ Since patients with eosinophilic bronchitis have higher concentrations of histamine and prostaglandin D₂ in their sputum than do patients with asthma,⁷ we hypothesized that there may be differences between the two conditions in the localization of mast cells within the airway wall. To test our hypothesis, we performed a comparative immunohistochemical analysis of bronchial-mucosa-biopsy specimens obtained from symptomatic patients with asthma, symptomatic patients with eosinophilic bronchitis, and normal controls.

METHODS**Study Subjects**

Subjects were recruited from two centers. A total of 15 subjects with asthma, 16 subjects with eosinophilic bronchitis, and 14 normal controls were recruited from Leicester, United Kingdom, and 15 subjects with asthma and 8 normal controls were recruited from Southampton, United Kingdom. There was assessable airway smooth muscle in the biopsy specimens from 8 subjects with asthma, 13 subjects with eosinophilic bronchitis, and 8 normal controls from Leicester and from 9 subjects with asthma and 3 normal controls from Southampton (Table 1). Subjects with asthma had characteristic symptoms and had variable airflow obstruction as indicated by one or more of the following: improvement by more than 15 percent in the forced expiratory volume in one second (FEV₁) 10 minutes after the inhalation of 200 μg of albuterol; airway hyperresponsiveness, defined by a provocative concentration of methacholine required to lower the FEV₁ by 20 percent (PC₂₀) of less than 8 mg per milliliter; or daily variability of more than 20 percent in the peak expiratory flow (PEF), as measured twice daily for 14 days. Subjects with eosinophilic bronchitis had a persistent isolated cough, no symptoms suggesting variable airflow obstruction during an observation period of at least two months, variability of less than 20 percent in the PEF, a normal chest radiograph, and on at least two occasions separated by more than two months, normal spirometric values, a PC₂₀ for methacholine of more than 16 mg per milliliter, and eosinophilia detectable in sputum (median percentage of eosinophils in sputum one week before bronchoscopy, 11.3 percent [range, 3.5 to 68.0]). Normal subjects were asymptomatic, had no evidence of variable airflow obstruction, and had a PC₂₀ for methacholine of more than 16 mg per milliliter. All subjects were currently nonsmokers with a smoking history of less than 10 pack-years. None of the subjects had taken inhaled or oral corticosteroids for at least six weeks before the study; all subjects with asthma used only short-acting β₂-adrenergic agonists as required. The study was approved by the Leicestershire

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TABLE 1. CLINICAL CHARACTERISTICS OF THE SUBJECTS.*

CHARACTERISTIC	SUBJECTS WITH EOSINOPHILIC BRONCHITIS (N=13)	SUBJECTS WITH ASTHMA (N=17)	NORMAL CONTROLS (N=11)
Age (yr)	45±12	40±15	33±17
Male sex (no.)	8	12	10
Atopy (no.)	7	14	2
PC ₂₀ for methacholine (mg/ml)			
Median	86	0.5	48
Range	18–128	0.03–4.6	16–128
FEV ₁ (% of predicted value)	101±11	100±16	102±18

*Plus-minus values are means ±SD. The PC₂₀ is the provocative concentration of methacholine required to lower the forced expiratory volume in one second (FEV₁) by 20 percent.

and Southampton ethics committees, and all subjects gave written informed consent.

Protocol and Clinical Measurements

The protocol required two visits one week apart. Spirometry, skin testing with allergens, and methacholine testing were performed, followed on recovery by the induction of sputum in the subjects at the Leicester site. Spirometry was performed with a dry bellows spirometer (Vitalograph), and the best of at least three successive readings within 100 ml of one another was recorded as the FEV₁. Skin tests were performed with *Dermatophagoides pteronyssinus*, cat dander, grass pollen, and *Aspergillus fumigatus* solutions, and normal saline and histamine were used as controls (Bencard). Subjects were considered to have a positive response to an allergen on skin testing if there was a wheal more than 2 mm larger in diameter than the negative control. The methacholine challenge was performed with the tidal-breathing method,⁸ with doubling concentrations of methacholine (from 0.03 mg per milliliter to 128 mg per milliliter); aerosols were generated with a Wright nebulizer. We extended the range of doses to include high doses in order to explore fully the dose–response relation. Sputum was induced and processed as previously described.⁹

At the second visit, bronchial-mucosa–biopsy specimens were obtained from the carinas of the right middle and lower lobes, according to the guidelines of the British Thoracic Society¹⁰ and with the use of local anesthesia and a fiberoptic bronchoscope (Olympus). All subjects received 2.5 mg of nebulized albuterol. Lidocaine (1 to 4 percent) was used for local anesthesia, and oxygen was given continuously through a nasal cannula during the procedure. Mucosal-biopsy specimens were immediately transferred into ice-cooled acetone containing the protease inhibitors iodoacetamide (20 mM) and phenylmethylsulfonylfluoride (2 mM) for fixation, stored at –20°C for 24 hours, and then processed into glycol methacrylate (Polysciences) for embedding.

Immunohistochemical Analysis

Two-micrometer sections were cut, placed in 0.2 percent ammonia solution for one minute, and dried at room temperature for one to four hours. The following monoclonal mouse IgG1 antibodies were used: anti-CD3 (Dako), AA1 antibody against mast-cell tryptase (Dako), and EG2 antibody against the cleaved form of eosinophil cationic protein (Pharmacia). In a subgroup of four

subjects with asthma, additional sequential sections were stained for mast-cell tryptase and chymase (Chemicon International) for colocalization. The technique of immunostaining applied to glycol methacrylate–embedded tissue has been described previously.¹¹ Briefly, slides were pretreated with a solution of 0.1 percent sodium azide and 0.3 percent hydrogen peroxide to inhibit endogenous peroxidase activity. After two 5-minute washes in TRIS-buffered saline (pH 7.6), a blocking medium consisting of Dulbecco's minimal essential medium, 10 percent fetal-calf serum, and 1 percent bovine serum albumin was applied for 30 minutes. Sections were then incubated with the primary antibody for 16 to 20 hours overnight at room temperature. Bound antibodies were labeled with biotinylated rabbit antimouse Fab fragments (Dako) during a two-hour incubation period and detected by the streptavidin–biotin–peroxidase method (Dako). Aminoethylcarbazole was applied as the chromogen, resulting in a red reaction product. Sections were counterstained with Mayer's hematoxylin. Appropriate control sections were similarly treated either without the primary monoclonal antibody or with an unrelated antibody of the same isotype (IgG1, Dako).

Assessment and Quantification

Areas of airway smooth muscle and subepithelial mucosa (lamina propria) were identified by morphologic examination, and the area was calculated with the use of a computer analysis system (Scion). We validated our detection of airway smooth muscle by comparing the area of smooth muscle measured in 11 pairs of contiguous sections after one of each pair of sections was assessed by morphologic examination and the other by staining for smooth-muscle actin (Dako). Nucleated, immunostained cells present in coded sections were enumerated in the lamina propria and airway smooth muscle, and the number of cells was expressed as the number per square millimeter of submucosa and smooth muscle. Cells were counted within the bundles of smooth muscle but not in the adjacent areas and were confirmed to be in the substance of the smooth muscle on the basis of serial sections obtained in order to avoid counting cells within the mucosal tissue that were juxtaposed owing to biopsy artifact. Tryptase-positive and chymase-positive mast cells within the airway smooth muscle were colocalized in a subgroup of four subjects with asthma who had sufficient airway smooth muscle. The thickness of the basement membrane and the lamina reticularis was calculated as the mean of 50 observations at 20- μ m intervals.¹²

Each of two observers who were unaware of the subjects' disease status examined 20 sections for the presence of airway smooth muscle. All specimens from the three groups were intermingled during processing and counting. A minimal area of 0.1 mm² from a biopsy section of airway smooth muscle was considered sufficient for the assessment of cellular infiltration, and two to four sections at least 10 μ m apart were assessed for each subject; these sections came from a single biopsy in 23 subjects and from two biopsies in 18 subjects. There were no differences between groups in the number of sections in which the area of smooth muscle was quantified. The area for each subject was expressed as the mean of the areas in all the assessable sections. In a single subject with asthma and two subjects with eosinophilic bronchitis, the basement membrane available for evaluation measured less than 1 mm in length, so data on the thickness of the basement membrane are not reported.

Statistical Analysis

Characteristics of the subjects are reported with the use of descriptive statistics. Cell counts are expressed as medians and ranges. Data on the thickness of the basement membrane and the lamina reticularis were normally distributed in each group, as confirmed by the Kolmogorov–Smirnov test for normality, and are reported as means ±SE. Comparisons among the three groups were made with the Kruskal–Wallis test; the Mann–Whitney U test was used for comparisons between groups involving nonparametric data;

and analysis of variance and unpaired t-tests were used for comparisons involving parametric data. Associations between the number of cells and the PC₂₀ for methacholine were established with the use of the Spearman rank-correlation method. The relation between the area of airway smooth muscle estimated by morphologic examination and that estimated by positive staining for smooth-muscle actin was expressed as the intraclass correlation coefficient. A P value of less than 0.05 was considered to indicate statistical significance. All tests were two-tailed.

RESULTS

The values for submucosal eosinophil counts, mast-cell counts, and thickness of the basement membrane and lamina reticularis are presented in Figure 1. The median submucosal eosinophil count was 2.1 per square millimeter (range, 0 to 12.4) in the normal controls, 9.5 per square millimeter (range, 2.5 to 75.0) in subjects with asthma, and 10.0 per square millimeter (range, 3.4 to 114.0) in subjects with eosinophilic bronchitis. There were significant differences in these counts between the controls and both the subjects with asthma (difference, 7.4 [95 percent confidence interval, 3.2 to 18.2]; $P=0.002$) and the subjects with eosinophilic bronchitis (difference, 7.9 [95 percent confidence interval, 4.0 to 18.7]; $P=0.002$), but there was no significant difference between the two groups with disease. There were no significant differences among the groups in the submucosal T-lymphocyte count (median among subjects with asthma, 48 per square millimeter [range, 22 to 122]; median among subjects with eosinophilic bronchitis, 42 per square millimeter [range, 9 to 145]; and median among normal controls, 53 per square millimeter [range, 8 to 255]; $P=0.53$) or the mast-cell count (median among subjects with asthma, 24 per square millimeter [range, 6 to 82]; median among subjects with eosinophilic bronchitis, 28 per square millimeter [range, 13 to 78]; and median among normal controls, 17 per square millimeter [range, 11 to 67]; $P=0.85$) (Fig. 1).

The mean (\pm SE) thickness of the basement membrane and the lamina reticularis was significantly greater in the subjects with asthma ($10.0\pm 0.5\ \mu\text{m}$) and those with eosinophilic bronchitis ($10.8\pm 1.4\ \mu\text{m}$) than in the normal controls ($6.7\pm 0.4\ \mu\text{m}$) (difference between subjects with asthma and normal controls, 3.3 [95 percent confidence interval, 1.9 to 4.7]; $P<0.001$; difference between subjects with eosinophilic bronchitis and normal controls, 4.1 [95 percent confidence interval, 0.9 to 7.2]; $P=0.02$), but there was no significant difference between the two groups with disease.

Smooth muscle could be readily identified by its morphologic appearance (Fig. 2A). The intraclass correlation coefficient between the area of smooth muscle measured by morphologic examination and that measured by positive staining for actin was 0.96. The area of smooth muscle assessed per biopsy section was similar in the three groups (median, $0.3\ \text{mm}^2$

in the subjects with asthma [range, 0.16 to 0.97]; $0.35\ \text{mm}^2$ in the subjects with eosinophilic bronchitis [range, 0.1 to 1.9]; and $0.3\ \text{mm}^2$ in the normal controls [range, 0.12 to 0.91]; $P=0.61$). In the subjects with asthma, there was a median of 2 mast cells per section of airway smooth muscle (range, 0 to 8); in the subjects with eosinophilic bronchitis, there was a median of 0 (range, 0 to 2); and in the normal controls, there was also a median of 0 (range, 0 to 5). The number of mast cells per square millimeter of smooth muscle was significantly higher in the subjects with asthma (median, 5.1 [range, 0 to 33.3]) than in subjects with eosinophilic bronchitis (median, 0 [range, 0 to 4.8]; difference, 5.1 [95 percent confidence interval, 2.5 to 6.1]; $P<0.001$) or in normal controls (median, 0 [range, 0 to 6.4]; difference, 5.1 [95 percent confidence interval, 0.2 to 5.9]; $P=0.01$) (Fig. 2B and 3).

There was a significant inverse correlation between the number of mast cells infiltrating the bronchial smooth muscle and the PC₂₀ for methacholine in the subjects with asthma ($r=-0.5$, $P=0.03$). In a subgroup of four subjects with asthma, 83 percent of the mast cells in airway smooth muscle were positive for tryptase and chymase. Eosinophils were observed in the airway smooth muscle in five subjects with asthma and in two subjects with eosinophilic bronchitis. T lymphocytes were only observed in two subjects with asthma and two normal controls. There were no significant differences between the atopic and nonatopic persons within a given group in terms of any of the measures we used.

DISCUSSION

Our results demonstrate that there is a striking difference between the number of mast cells in the airway smooth muscle in patients with asthma and the number in both normal subjects and patients with eosinophilic bronchitis. This observation has potential implications for our understanding of the pathogenesis of asthma and the pathophysiological role that mast cells have in this disease.

It is possible that mast-cell infiltration of the airway smooth muscle is a general feature of obstructive lung disease and is not specific to asthma. One limitation of our study is the absence of a control group of subjects with other obstructive lung diseases such as chronic obstructive pulmonary disease, although given the overlap between the clinical and pathophysiological features of asthma and chronic obstructive pulmonary disease,¹³ such studies would have to be interpreted with careful attention to clinical phenotype.

We used subjects with eosinophilic bronchitis as a control group with disease because eosinophilic bronchitis shares many of the features of asthma but is characterized by normal airway function.⁴ We reasoned that any difference in pathology between the

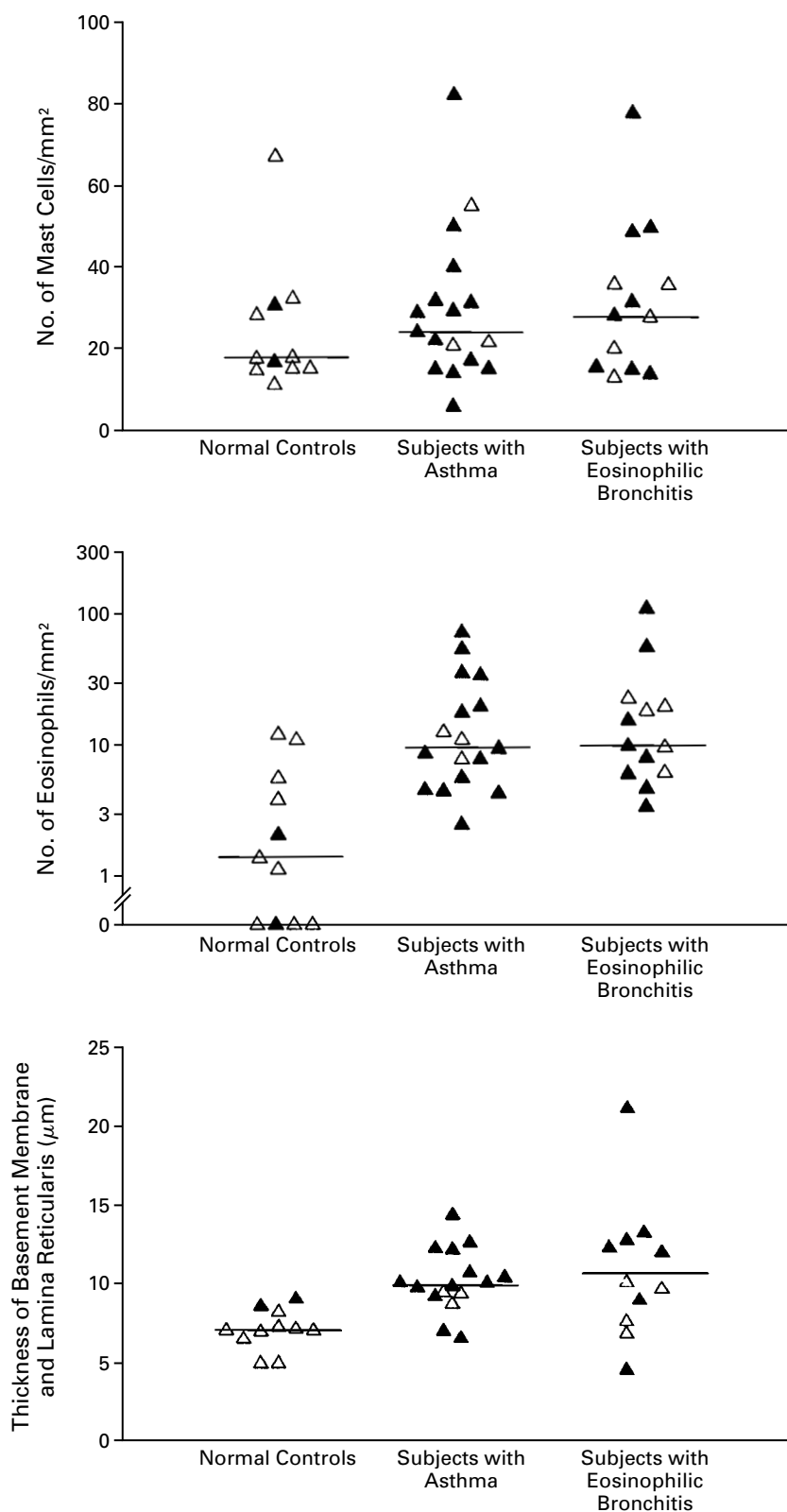


Figure 1. Numbers of Tryptase-Positive Submucosal Mast Cells and EG2-Antibody-Positive Submucosal Eosinophils per Square Millimeter and Thickness of the Basement Membrane and Lamina Reticularis in Subjects with Asthma, Subjects with Eosinophilic Bronchitis, and Normal Controls.

For the eosinophil count, $P=0.001$ by the Kruskal–Wallis test for the comparison among the three groups. For the thickness of the basement membrane and the lamina reticularis, $P=0.002$ by analysis of variance for the comparison among the three groups. Solid triangles represent atopic subjects, and open triangles nonatopic subjects. The horizontal lines represent the median values in the top two panels and the mean value in the bottom panel.

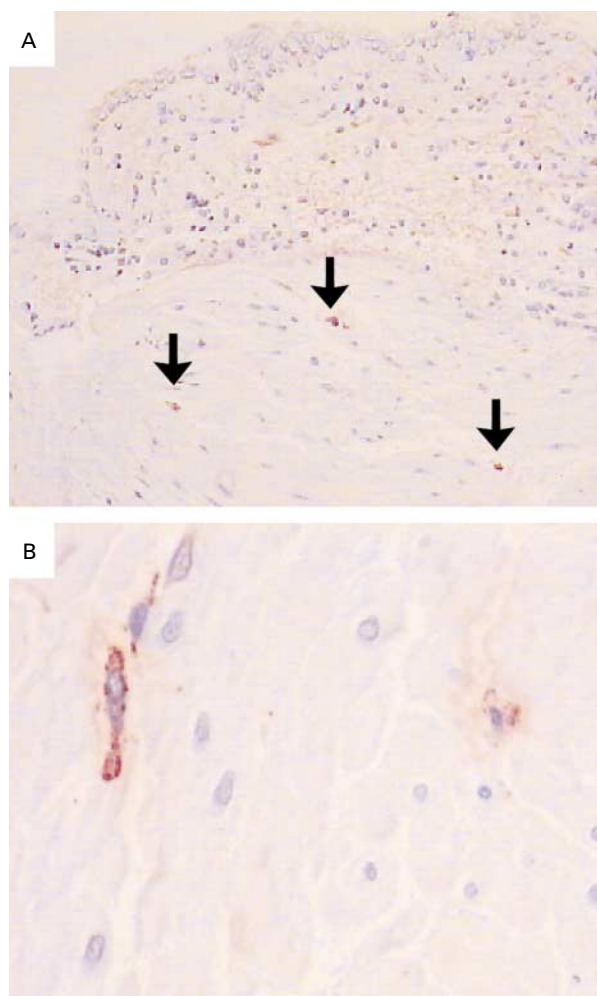


Figure 2. Bronchial-Biopsy Specimens from Subjects with Asthma.

Panel A shows epithelium, submucosa, and smooth muscle with mast cells (arrows) infiltrating the airway smooth muscle ($\times 100$). Panel B shows mast cells within the airway smooth muscle (hematoxylin, $\times 400$).

two conditions would most likely be related to the features that are relevant to these functional abnormalities. We observed a striking difference between the number of mast cells that were present in the airway smooth muscle of subjects with asthma and the number in either normal subjects or subjects with eosinophilic bronchitis.

The hypothesis that mast cells are localized in the airway smooth muscle and that interactions between mast cells and smooth-muscle cells are important in asthma is plausible. Airway smooth muscle can pro-

vide the correct microenvironment for the differentiation, activation, and survival of mast cells.¹⁴ Several mast-cell products have the potential to affect adversely the growth and function of smooth muscle, and their microlocalization in the smooth muscle would probably facilitate this interaction. For example, the mast-cell-derived autacoid mediators histamine, prostaglandin D₂, and the cysteinyl leukotrienes are potent spasmogens of airway smooth muscle, and the mast-cell-specific serine protease tryptase could potentially induce bronchoconstriction, airway remodeling, and airway hyperresponsiveness through a variety of mechanisms.¹⁴⁻¹⁷ The hypothesis that the infiltration of mast cells into airway smooth muscle is functionally important is supported by our observation that the number of mast cells in the smooth muscle of patients with asthma was inversely correlated with the degree of airway hyperresponsiveness.

We have previously found greater concentrations of the mast-cell products histamine and prostaglandin D₂ in induced sputum from subjects with eosinophilic bronchitis than in sputum from subjects with asthma.⁷ Furthermore, the number of mast cells in bronchial brushings was significantly higher in subjects with eosinophilic bronchitis than in those with asthma.⁶ These observations suggest that mast cells might preferentially localize in the superficial airway structures in patients with eosinophilic bronchitis. The assessment of airway epithelium by means of bronchial biopsies is confounded by variation in epithelial integrity, which may reflect a real effect of disease or an artifact¹⁸; therefore, the biopsy material collected in our study is inadequate for testing this hypothesis. Further studies using a wider variety of techniques to sample the lower airway are required in order to explore in greater detail the localization of mast cells within the airway in patients with eosinophilic bronchitis and patients with asthma.

Most inflammatory mediators are rapidly inactivated once they leave the cell, so they act across distances of only a few micrometers. Microlocalization is therefore a fundamental organizing principle of inflammatory responses, although it has not been given sufficient attention in previous studies of the immunopathology of asthma — in part because thick frozen sections do not provide adequate detail for morphologic examination and only small amounts of smooth-muscle tissue can be obtained by fiberoptic bronchoscopy. With the use of glycol-methacrylate embedding and ultrathin sections, we obtained excellent samples for morphologic examination. The identification of smooth muscle was validated by means of actin staining and was found to be reliable. Although relatively small amounts of smooth muscle were present, we believe our data are robust. There were no differences among the three groups in the

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