

Respiratory pathophysiologic responses

Inflammation of small airways in asthma

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This study was designed to examine the inflammatory process in the central and peripheral airways of surgically resected lungs from asthmatic and nonasthmatic subjects. Lung specimens were inflated with cryoprotective, rapidly frozen, and systematically sampled. Cryosections prepared from frozen tissue blocks were fixed in acetone/methanol and immunostained with monoclonal antibodies by using the alkaline phosphatase-anti-alkaline phosphatase technique to detect CD3 (T cells), major basic protein (total eosinophils), EG2 (activated eosinophils), anti-tryptase (mast cells), anti-elastase (neutrophils), and CD68 (macrophages). All airways from patients with asthma demonstrated a significant increase in the numbers of T cells and total and activated eosinophils compared with airways from nonasthmatic subjects ($p < 0.001$). In the patients with asthma, the numbers of activated eosinophils but not T cells were significantly greater in airways with an internal perimeter less than 2 mm compared with those with an internal perimeter greater than 2 mm ($p < 0.05$). There were also significantly higher numbers of major basic protein-positive eosinophils, when expressed as a fraction of the alveolar wall tissue, in patients with asthma compared with control subjects ($p < 0.05$). In asthmatic airways with an internal perimeter of more than 2 mm, there was a greater number of activated eosinophils in the tissue between the epithelium and the smooth muscle compared with the tissue between the smooth muscle layer and lung parenchyma ($p < 0.05$). In contrast, there was a greater number of total eosinophils in the outer airway layer compared with the inner airway layer ($p < 0.05$). These results show that there is a similar but more severe inflammatory process present in the peripheral compared with the central airways of patients with asthma, which is consistent with the fact that the smaller airways are a major site of obstruction in asthma. (*J Allergy Clin Immunol* 1997;100:44-51.)

Key words: Asthma, small airways, airway wall, inflammation, eosinophils

Asthma is characterized by episodic airway obstruction and increased bronchial responsiveness to the inhalation of nonspecific irritants.^{1,2} The concept that these

Abbreviations used

MBP: Major basic protein
MMF: Mid-maximal expiratory flow rate
Pi: Internal perimeter

abnormalities are related to an inflammatory process in the conducting airways was established more than 100 years ago.³ Since then, a number of postmortem studies have provided evidence in support of the concept that airway inflammation is the key process in asthma,⁴⁻⁶ and the introduction of fiberoptic bronchoscopy and the application of immunocytochemistry and in situ hybridization to small biopsy specimens and lavage fluid has allowed this inflammatory process to be studied in more detail.⁷⁻⁹ Studies in which direct measurements of bronchial pressure and flow were used have established the peripheral airways as the major site of airway obstruction in patients with asthma.¹⁰ This conclusion was supported by a computational analysis based on quantitative histology, which showed that the peripheral rather than the central airways account for the majority of the airway hyperresponsiveness in this condition.^{11,12}

Bronchoscopic studies have clearly shown that asthma is associated with infiltration of the major airways with chronic inflammatory cells, particularly lymphocytes and eosinophils,^{7,8} and the upregulation of a T_H2-type cytokine profile.¹³⁻¹⁶ However, studies that use bronchoalveolar lavage and/or endobronchial biopsy specimens are limited in the amount and type of information they can yield regarding the inflammatory process in the smaller airways, bronchioles, and alveoli. Although postmortem studies clearly show that the entire length of the airway is involved in asthma,^{4-6,12} there have been no systematic studies of the peripheral airways in living patients similar to those provided by fiberoptic bronchoscopy of the central airways. The purpose of this study was to examine the small airways and parenchyma of resected lung specimens from patients with asthma and nonasthmatic control subjects and to compare the changes in central and peripheral airways by using immunocytochemistry to identify the inflammatory cells present in the tissue. We hypothesized that the number of eosinophils and lymphocytes is increased throughout the airways of patients with asthma and that this change is predominantly in the peripheral airways.

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TABLE I. Pulmonary function data in patients with asthma and nonasthmatic control subjects

	Patients with asthma (n = 6)	Nonasthmatic subjects (n = 10)
Age (yr)	55.0 ± 15.5	57.9 ± 14.4
Sex (M:F)	4:2	6:4
Cigarettes (pack-years)	40.8 ± 31.9	41.9 ± 26.8
TLC (% predicted)	103.9 ± 1.9	107.0 ± 10.2
FVC (% predicted)	96.6 ± 29.7	114.3 ± 19.5
RV (% predicted)	110.6 ± 47.2	114.4 ± 23.0
FEV ₁ (% predicted)	82.5 ± 22.1	89.2 ± 15.3
MMF (% predicted)	56.3 ± 30.1	65.5 ± 24.7
K _{CO} (% predicted)	66.4 ± 17.8	70.7 ± 9.5

Values are expressed as mean ± SD.

TLC, Total lung capacity; FVC, forced vital capacity; RV, residual volume; K_{CO}, Transfer factor of carbon monoxide.

METHODS

Subjects

Lung tissue was obtained from 16 subjects selected from a group of 108 patients who entered the St. Paul's Hospital Lung Study between 1991 and 1995, for which ethical approval was obtained. This ongoing study of lung structure and function is based on a prospective study of pulmonary function in patients requiring lung resection for treatment of carcinoma. Lung function was measured in the immediate preoperative period. A clinical diagnosis of asthma was made in six of these 108 patients in the present series on the basis of an evaluation of the patients' medical files by a respiratory physician. The clinical criteria used to establish this diagnosis included prior physician diagnosis and treatment for asthma and documented evidence of variable airflow obstruction greater than 15% and bronchial hyperresponsiveness. Although three of the six patients with asthma who were selected had received inhaled steroid treatment for their asthma, only two had been receiving steroid treatment at the time of their lung resection. Ten control subjects who did not demonstrate these features were selected from the same group of 108 patients on the basis of the best match to the asthmatic group with respect to age, sex, smoking history, and lung function.

Tissue preparation

All lung specimens used in the study were taken from the operating room immediately after resection and allowed to collapse. Each tumor was palpated through the pleural surface, transected, and sampled for histologic examination. The hilar lymph nodes were dissected, and the bronchial resection margin was sampled to obtain the tissue required for the surgical pathology report. The pleural surface was then sutured shut, and the lung was inflated through the bronchi with optimal cutting temperature solution diluted 50% with normal saline to decrease viscosity. The inflated specimen was then frozen in an insulated box over liquid nitrogen, maintained at dry ice temperature, and cut into 2 cm thick transverse slices by using a band saw. These lung slices were kept on dry ice until they were sampled with the use of a power-driven drill with a hollow bit to obtain cores of tissue 1.5 cm in diameter to 2 cm in length. The frozen samples of tissue were stored at -70° C until their subsequent use, and the remaining lung tissue was placed in buffered

TABLE II. Number and distribution of airways analyzed in patients with asthma and nonasthmatic control subjects

	Patients with asthma (n = 6)	Nonasthmatic subjects (n = 10)
Cores	1.3 ± 0.2	1.7 ± 0.2
Airways	2.7 ± 0.7	4.4 ± 0.5*
Airways/core	2.0 ± 0.3	2.6 ± 0.2
Pi of total airways (mm)	3.1 ± 0.5 (n = 6 [16])	4.0 ± 0.7 (n = 10 [44])
Pi of airways <2 mm (mm)	1.4 ± 0.5 (n = 4 [6])	1.4 ± 0.1 (n = 5 [14])
Pi of airways >2 mm (mm)	3.5 ± 0.4 (n = 6 [10])	4.6 ± 0.6 (n = 10 [30])

Values are expressed as means ± SEM. Values in brackets within parentheses indicate the total number of airways sampled.

*p < 0.05.

formalin for 24 hours, after which it was further sampled and processed for light microscopy with standard techniques.

Conventional histology and immunocytochemistry

Twenty-five cores of frozen lung tissue were selected from the 16 study subjects (8 cores of tissue from 6 patients with asthma and 17 from control subjects) and shipped to the Meakins-Christie Laboratories (McGill University, Montreal, Quebec, Canada) with a code to present the tissue in a random fashion. From these cores of tissue, 6 µm cryostat serial sections were cut, fixed in 60:40 acetone/methanol for 10 minutes, air dried, and stained with hematoxylin and eosin for conventional histologic examination; sections were also immunostained for different inflammatory cell markers by using the alkaline phosphatase-anti-alkaline phosphatase method. The monoclonal antibodies used were CD3 (pan T lymphocyte, Becton-Dickinson), major basic protein (MBP) (total eosinophils, a gift from Dr. R. Moqbel), EG2 (activated eosinophils secreting eosinophil cationic protein, Kabi Pharmacia), anti-tryptase (mast cells, Chemicon), anti-elastase (neutrophils, Dako), and CD68 (macrophages, Dako). Briefly, cryostat sections were incubated with primary monoclonal antibody overnight at 4° C, washed, and treated with rabbit anti-mouse antibody for 30 minutes at room temperature. The antibody-antigen complex was developed with the alkaline phosphatase-anti-alkaline phosphatase complex and Fast Red (Sigma Chemical Co.). Separate sets of sections were treated with normal IgG as negative controls.

Quantification of immunoreactive cells in the airway wall

The internal perimeter (Pi) of all airways identified in the sections was measured by using an National Institutes of Health image analysis system.¹⁷ Immunoreactive cells present in the airway wall were counted and expressed as number of positive cells per square millimeter of the airway wall. When possible, the immunoreactive cells were also counted separately in the inner airway wall between the basement membrane and the smooth muscle (A1) and in the outer wall of the tissue extending from the outer border of the smooth muscle to the parenchyma (A2). To determine the numbers of immunoreactive cells as a fraction of the alveolar tissue, a point counting technique was used as previously described.¹⁸ Briefly, a point

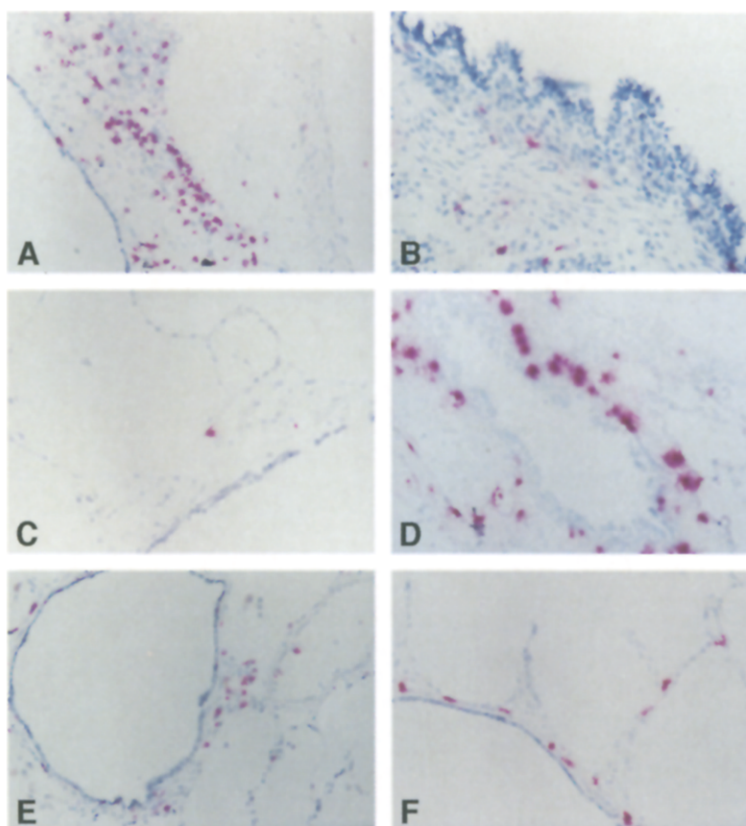


FIG. 1. Representative examples of immunocytochemical identification of T cells (CD3) (A) and MBP-positive eosinophils (B) in airways greater than 2 mm in diameter from patients with asthma. C, Example of MBP immunostaining of an airway greater than 2 mm in diameter from a nonasthmatic subject. D, Example of EG2-positive cells (activated eosinophils) in an airway less than 2 mm in diameter. Note the large number of activated eosinophils present. E, Example of T-cell immunostaining in asthmatic airways less than 2 mm in diameter. F, Example of eosinophil MBP immunostaining in lung parenchyma tissue from a patient with asthma.

count was made of each entire section of lung tissue at low magnification to calculate the proportion of points that fell on airspace, alveolar wall tissue, and large vessel tissue. This then allowed us to express the proportion of the tissue sample made up of each of these elements. The airways were then examined at a higher magnification ($\times 100$), and each of the areas was sampled by using an ocular equilateral triangle grid (Wiebel type 2). The sum of all points on immunoreactive positive cells (activated and total eosinophils, neutrophils, T cells, mast cells, macrophages) was then expressed as the sum of all points falling on alveolar wall tissue, and the volume fraction was then calculated. When all of the data were obtained, they were shipped to Vancouver (Pulmonary Research Laboratory, University of British Columbia, St. Paul's Hospital, Vancouver, Canada) where the code was broken.

Statistics

Comparisons between cell counts in the patients with asthma and normal control subjects were performed by using a non-parametric Mann-Whitney U test. Comparisons of different cell types within patients with asthma were obtained by using the Wilcoxon signed-rank test. Probability (p) values of less than 0.05 were considered statistically significant.

RESULTS

Table I provides the pulmonary function data for all of the patients, showing that both the asthmatic and control groups had approximate 40 pack-year histories of smoking and were well matched for age, sex, and lung function. The patients with asthma ranged in age from 33 to 77 years (55 ± 15 years), their preoperative FEV₁ ranged from 52% to 112%, and the maximum mid-expiratory flow rate (MMF) ranged from 47% to 104% of the predicted values. The control subjects ranged in age from 25 to 74 years (57 ± 14 years), and their FEV₁ and MMF ranged from 57% to 105% and 22% to 95% of the predicted values, respectively.

The histologic studies showed that the Pi of the airways varied from 0.8 to 16.4 mm with a similar frequency distribution of size between the asthmatic and control lungs (Table II). Immunoreactive cells infiltrating the airway wall were enumerated in different-sized airways and were expressed per square millimeter of tissue (Fig. 1). All airways (<2 mm Pi and >2 mm Pi) from patients with asthma demonstrated an increase in the number of T cells (CD3), activated eosinophils

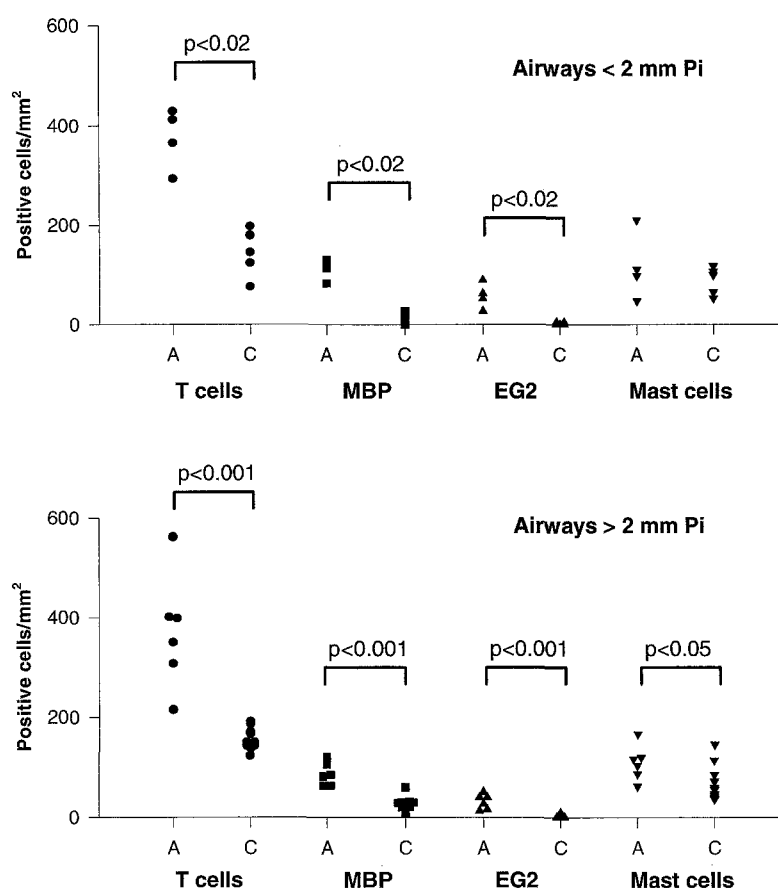


FIG. 2. Immunocytochemical cell markers in airways less than 2 mm in diameter (**A**) and airways greater than 2 mm in diameter from patients with asthma (**B**) and control subjects. T cells (CD3), total eosinophils (MBP), activated eosinophils (EG2), and mast cells (tryptase) were studied. Values given are average data of six (<2 mm) or 10 (>2 mm) airways from four to six patients with asthma and 13 (<2 mm) or 31 (>2 mm) airways from six to 10 nonasthmatic control subjects. A, Patients with asthma; C, control subjects.

(EG2), and total eosinophils (MBP) compared with those from nonasthmatic subjects (Fig. 2, $p < 0.001$). The number of mast cells (anti-tryptase) was significantly greater in airways (>2 mm Pi) of patients with asthma compared with those of control subjects (Fig. 2, B , $p < 0.05$). However, there were no significant differences in the numbers of macrophages (CD68, mean $\pm$ SEM: patients with asthma [$n = 6$], 140.8 ± 12.5 ; control subjects [$n = 10$], 144.5 ± 10.8 ; $p > 0.05$) and neutrophils (elastase, mean $\pm$ SEM: patients with asthma [$n = 6$], 61.8 ± 2.9 ; control subjects [$n = 10$], 72.9 ± 7.6 ; $p > 0.05$) in the airways of the patients with asthma compared with the control subjects. In the patients with asthma, the numbers of activated eosinophils but not T cells were significantly greater in airways with a Pi of less than 2 mm compared with airways with a Pi greater than 2 mm (Fig. 3, $p < 0.05$).

In airways with a Pi greater than 2 mm, immunoreactive cells were quantified in tissue between the epithelial layer and the smooth muscle layer (A1) and also in tissue beyond the smooth muscle layer up to adjacent paren-

chyma (A2), as shown in Fig. 4. This analysis was performed on 10 airways from patients with asthma and 15 airways from nonasthmatic control subjects. There were no significant differences in each of the two groups in the number of cells that were immunoreactive for CD3, CD68 elastase, and mast cells between the two compartments. However, in the patients with asthma, there was a greater number of total eosinophils in the outer airway layer than in the inner airway layer (Fig. 5, $p < 0.05$) and a significantly greater number of activated eosinophils in the inner airway layer compared with the outer airway layer ($p < 0.05$). In the nonasthmatic control subjects, there were no significant differences between the inner and outer airway compartments for any of the cell types examined ($p > 0.05$).

The number of MBP-positive eosinophils presented as a volume fraction of the alveolar wall tissue was greater in patients with asthma than in control subjects (mean $\pm$ SEM: patients with asthma [$n = 6$], 2.5 ± 0.3 ; control subjects [$n = 10$], 1.2 ± 0.1 ; $p < 0.05$). For all other cells studied, there were no significant differences between

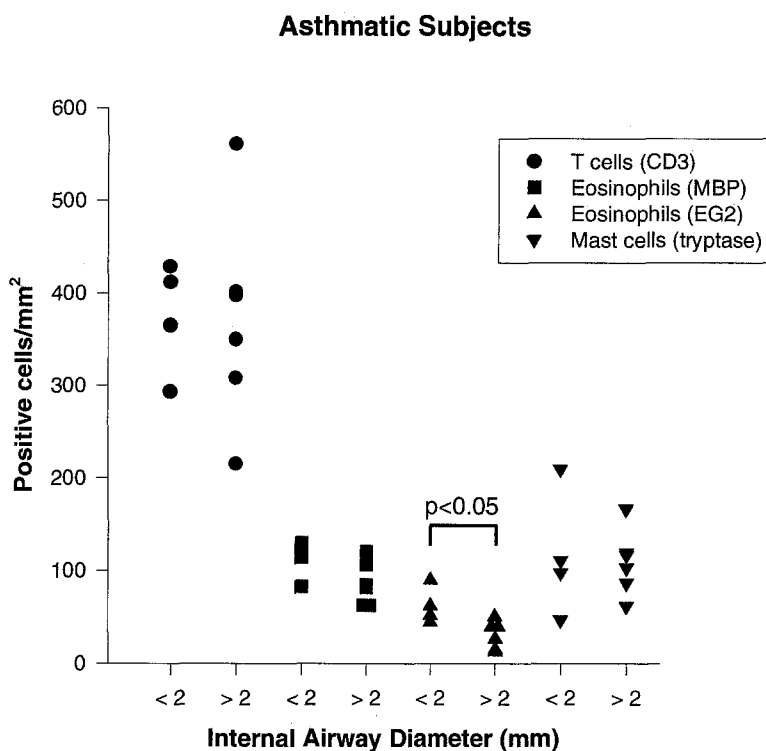


FIG. 3. Immunocytochemical cell markers in airways less than 2 mm and greater than 2 mm in diameter from patients with asthma. T cells (CD3), total eosinophils (MBP), activated eosinophils (EG2), and mast cells (tryptase) were examined. For airways with a Pi of less than 2 mm, values given are means of six airways from four patients with asthma; for airways with a Pi of greater than 2 mm, values given are means of 10 airways from six patients with asthma.

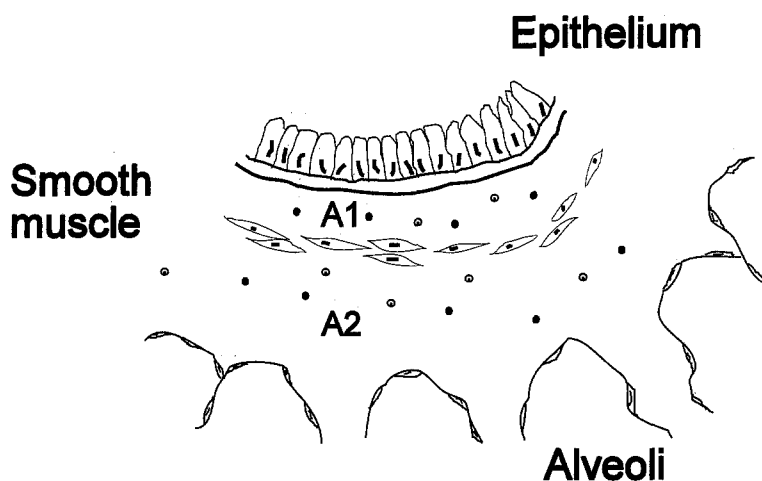


FIG. 4. Schematic diagram showing tissue area between epithelial layer and smooth muscle layer (A1) and between smooth muscle layer and adjacent parenchyma (A2).

the patients with asthma and the nonasthmatic control subjects when the number of immunoreactive cells was presented as a fraction of the alveolar wall tissue ($p > 0.05$). There was no correlation between the number of eosinophils or T cells and FEV_1 in the patients with asthma ($p > 0.05$).

DISCUSSION

This study demonstrates an increase in the number of T lymphocytes and eosinophils in the airways of patients with asthma compared with nonasthmatic control subjects matched for age, sex, smoking history, lung function, and airway size. The presence of these inflamma-

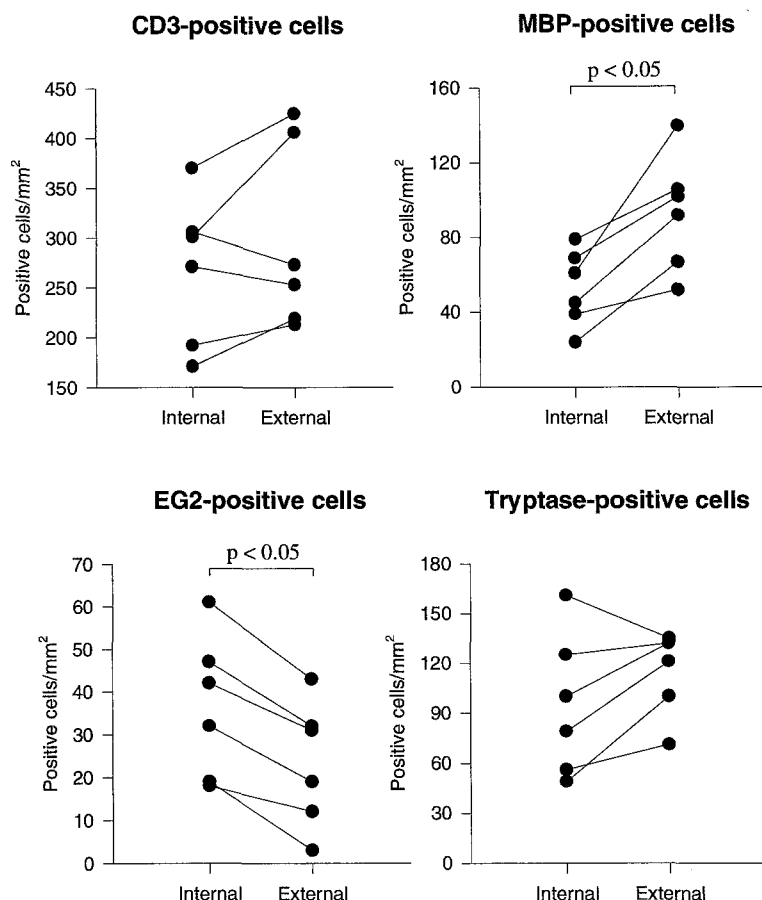


FIG. 5. Distribution of T cells (CD3), total eosinophils (MBP), activated eosinophils (EG2), and mast cells (tryptase) in A1 (internal layer) and A2 (external layer) in the airways of patients with asthma. Values given are means of 10 airways from six patients with asthma.

tory cells in larger airways is consistent with the increased numbers of T lymphocytes and eosinophils reported from bronchoscopic biopsy specimens of asthmatic airways.^{8, 9, 14, 16} The observed differences in inflammatory cells between the asthmatic and nonasthmatic groups, despite the cigarette-related inflammatory changes in both groups and the presence of relatively mild asthma, is consistent with previous reports of the presence of mucosal inflammation in patients with newly diagnosed asthma.¹⁹ However, our results also extend the biopsy findings by showing that a similar but more severe inflammatory process is present in peripheral airways not routinely biopsied during fiberoptic bronchoscopy.

Expressing cells per millimeter of basement membrane underestimates the total number of inflamed cells present in the airways less than 2 mm in diameter because the total perimeter of all of the smaller airways is much greater than that of central airways because of the rapid increase in both number and total cross-sectional area in the peripheral conducting airways. Indeed, calculations based on a computerized model of the airways¹¹ show that the small but consistent increase in total and activated eosinophil counts

per millimeter of basement membrane in the peripheral airways compared with the central airways, shown in Fig. 3, should be increased by a factor of approximately 100 to take this fact into account. The presence of an enhanced inflammatory process in the small airways of these patients with asthma is consistent with the measured increase in peripheral airway resistance in patients with asthma¹⁰ and with the prediction that the peripheral airways are the site of the increased airway responsiveness in patients with asthma.^{11, 12}

An increased number of inflammatory cells in the smaller airways in asthma has therapeutic, as well as diagnostic, implications. Inhaled steroid therapy has been shown to decrease inflammation in the proximal airways^{20, 21} but may not be as efficient in the smaller airways. Although it could be argued that the two patients with asthma in the study who had been receiving inhaled steroids at the time of their lung resection surgery may have biased us to find increased inflammatory cells in the peripheral airways, when our data was re-analyzed with these two subjects excluded, all the initial findings remained highly statistically significant. This suggests that chronic and relatively asymptomatic

inflammation may be present in the peripheral airways of patients with asthma treated with or without inhaled steroids. Such inflammation could then result in fibrotic changes and fixed airway obstruction in the peripheral lung in the later stages of this disease.

Bronchoscopic sampling is not only limited to proximal airway sites but is also limited to the depth of the submucosa. In the airways that were greater than 2 mm in diameter, our results show that the T-cell infiltrate in the submucosa is a reliable marker of the number of T cells infiltrating the whole airway wall. However, because the T cells were not examined for activation markers, they may be different in their functional phenotype, as previously reported.^{9, 13, 15, 22, 23} This possibility is supported by the finding that there was an increased number of total eosinophils in the outer airway layer, as well as an increase in the number of activated eosinophils in the inner airway layer, a finding that also suggests that the submucosa may be the predominant site of asthmatic inflammation because it is the major site of particulate antigen deposition.

The increased number of MBP-positive eosinophils in the alveolar wall tissue in the lungs of patients with asthma compared with those of control subjects shows that the inflammatory process present in the peripheral lungs of patients with asthma is based on both the bronchial and pulmonary circulations. This observation is consistent with the fact that this lung region is the major site of anastomoses between these two vascular beds. The presence and activation of eosinophils is the most striking result of our study and adds to the already existing evidence that the eosinophil is important in asthma.²⁴⁻²⁷ The lack of correlation between eosinophils and FEV₁ or MMF in our results is attributed to the contribution of smoking to airflow limitation in both groups of subjects and the need for a larger group of subjects to obtain the power to show an effect of the asthmatic changes over and above that caused by smoking.²⁸

Although there were no differences in the number of T cells that were present in the alveoli between the asthmatic and nonasthmatic groups, there may still be a difference in their phenotype, especially with respect to the production of cytokines such as IL-5, which would activate eosinophils,^{13, 29, 30} or interferon- γ , which would downregulate a T_{H2} response.^{14, 31}

The inflammatory process present in the peripheral airways of these patients with asthma could contribute to an increase in airway resistance in several ways. The replacement of the surfactant present in the luminal surface of these airways by an inflammatory exudate would contribute to early closure and gas trapping.³² An increase in the volume of tissue between the epithelium and muscle would amplify the effect of airway smooth muscle shortening on the caliber of the airways, and the inflammatory changes in the outer wall of the airways would interfere with the load that the parenchyma normally places on the smooth muscle of airways to prevent it from shortening.¹¹ The way in which the

inflammatory cell population demonstrated here contributes to these structural and functional changes remains to be determined.

In summary, these studies demonstrate that the T-cell accumulation and eosinophil activation associated with the asthmatic state is dominant in the smaller bronchi and bronchioles and suggest that the inflammatory response at this airway site is a key feature of the pathophysiology of asthma.

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