

METHODS

Subject selection and evaluation

Twelve subjects 21 to 35 years of age with a history of recurrent seasonal pollen-induced rhinitis but no past or current history of asthma took part in the study. All had positive allergic skin test responses to tree, grass, and/or ragweed pollen. Six subjects were also sensitized to house dust, although they had minimal symptoms out of the pollen season. All subjects were nonsmokers who had no other respiratory conditions apart from their allergic rhinitis, and none had used anti-inflammatory drugs, such as nasal, inhaled, or oral steroids or β_2 -agonists. Short-acting antihistamines and nasal steroids were allowed after the in-season bronchoscopy. Our institutional ethics committee approved the study, and all subjects gave their informed consent.

The subjects were studied out of the pollen season (mid-September to December) and at the peak period of allergic rhinoconjunctivitis (June to September). Spirometry and airway responses to inhaled methacholine were assessed by using standardized procedures in and out of the pollen season.¹⁹ All subjects had a methacholine PC₂₀ value of greater than 8 mg/mL²⁰ in and out of the pollen season.

Bronchoscopy and bronchial biopsy specimens

The subjects showed no evidence of respiratory infection for at least 4 weeks before the bronchoscopy. The bronchoscopy procedures were performed in and out of the pollen season under oxygen at 5 min·mL⁻¹ given by nasal catheter. After local anesthesia of the throat, larynx, and bronchi was obtained by using 2% or 4% xylocaine, a flexible bronchoscope (Olympus OES 10 fiberscope, Olympus, Optical Co Ltd) was introduced into the bronchial tree, and 4 to 6 tissue samples were removed from the carinae of the lobar and segmental bronchi of different lungs on two occasions by using conventional forceps. Vital signs and electrocardiograph and oximetry readings were monitored throughout the procedure. No bronchodilator was administered before or after the bronchoscopic procedure.

Immunohistochemistry

The biopsy specimens were immediately placed in acetone containing the protease inhibitors phenylmethylsulfonyl fluoride and iodoacetamide (each at 2 mmol/L, Sigma Chemical Co) and cooled rapidly to -20°C. They were transferred to acetone for 15 minutes and then to methylbenzoyl for another 15 minutes. After this, the biopsy specimens were immersed in glycolmethacrylate (GMA) monomer (Polyscience) at 4°C for 7 hours. During this time, the GMA solution was changed 3 times. Finally, the tissue was embedded in GMA resin prepared by mixing GMA monomer and N,N-dimethylalanine in PEG 400 and benzoyl peroxide and polymerized overnight at 4°C.²¹ Two-micrometer sections were cut by using an ultramicrotome (Leica Reichert Ultracut S) and floated on water containing 0.02% ammonia until fixed on poly-L-lysine-coated slides.

The following antibodies (dilutions) were used: mouse anti-human CD3 (1:5), CD25 (1:100), CD45RO (1:50), AA1 (tryptase) (1:100), and HLA-Dr (1:400) from Dako/Dimension Laboratories; CD4 (1:25) and CD8 (1:50) from Becton Dickinson; anti-EG1 (1:100) and EG2 (1:200) from Kabi Pharmacia Diagnostics; anti-IL-5 (1:50) from Genzyme; and anti-IL-4 (1:100) and anti-IFN- γ (1:100) from Cederlanes.

Bronchial biopsy immunostaining was performed as previously described.¹⁶ Briefly, endogenous peroxidase background activity was blocked by incubation with 0.3% H₂O₂ plus 0.1% sodium azide in TRIS-buffered saline for 30 minutes and then in normal serum for 30 minutes. The samples were then incubated overnight with the primary antibody (4°C), for 1 hour with a biotinylated secondary antibody (23°C), and for 40 minutes with ABC-HRP complex (23°C). The slides were washed 3 times with TRIS-buffered saline

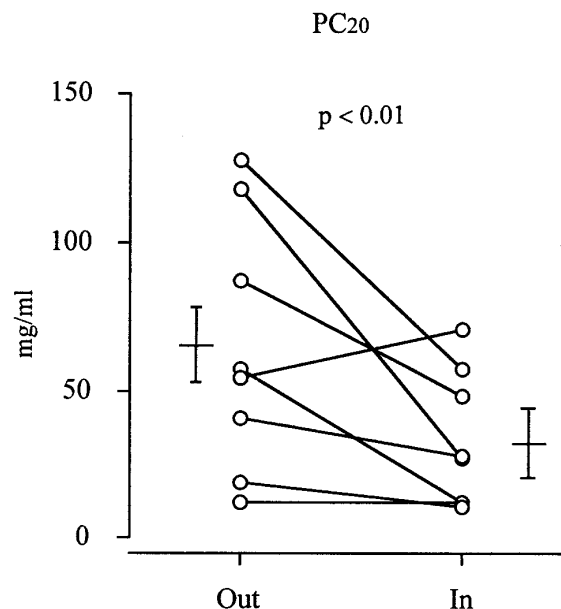


FIG 1. Seasonal variation in PC₂₀ values of nonasthmatic subjects with rhinitis. Airway responsiveness to methacholine increased significantly in the pollen season compared with the out-of-season period. Bars indicate geometric means $\pm$ SEM.

between each step. To detect bound antibodies, the slides were incubated with amino-ethylcarbazole substrate until staining was visible under a microscope. The reaction was stopped by rinsing with water, and the biopsy specimens were counterstained with Mayer Hematoxylin. The slides were mounted with Crystal Mount (Bio-medex). Irrelevant isotypic antibodies were used as negative controls.

Quantification of immunohistochemical staining

All slides were counted in a blind manner by using an Eclipse E-600 Nikon microscope (Nikon). Two tissue sections were counted. Mucosal areas were measured with a calibrated image analysis system (Mocha image analysis software, Jandel Scientific), and specimens under 0.2 mm² were automatically excluded. Counts were expressed as the number of positive cells per square millimeter of bronchial connective tissue, excluding mucus glands, blood vessels, and smooth muscles.

Electron microscopy

Electron microscopic samples were fixed in Karnovsky's fluid, washed in cacodylate buffer, osmicated, dehydrated in alcohol, and embedded in Epon. Half of the samples were treated en bloc with uranyl acetate. All sections were stained with lead citrate and analyzed with a Jeol 100 CX electron microscope. The electron microscopic studies evaluated mast cell and eosinophil degranulation.

Statistical analysis

Results are presented as means $\pm$ SEM. PC₂₀ geometric mean values were calculated for each group. If the PC₂₀ value was greater than 128 mg·mL⁻¹, it was considered to be 128 mg·mL⁻¹. A randomized block design (ANOVA) was applied to the comparison between in- and out-of-pollen-season samples. Spearman regression analyses were used to measure relationships between variables. Normality and variance assumptions measured by using SAS software were fulfilled for the parameters studied except for CD4, which was analyzed by using the Wilcoxon signed-rank test.

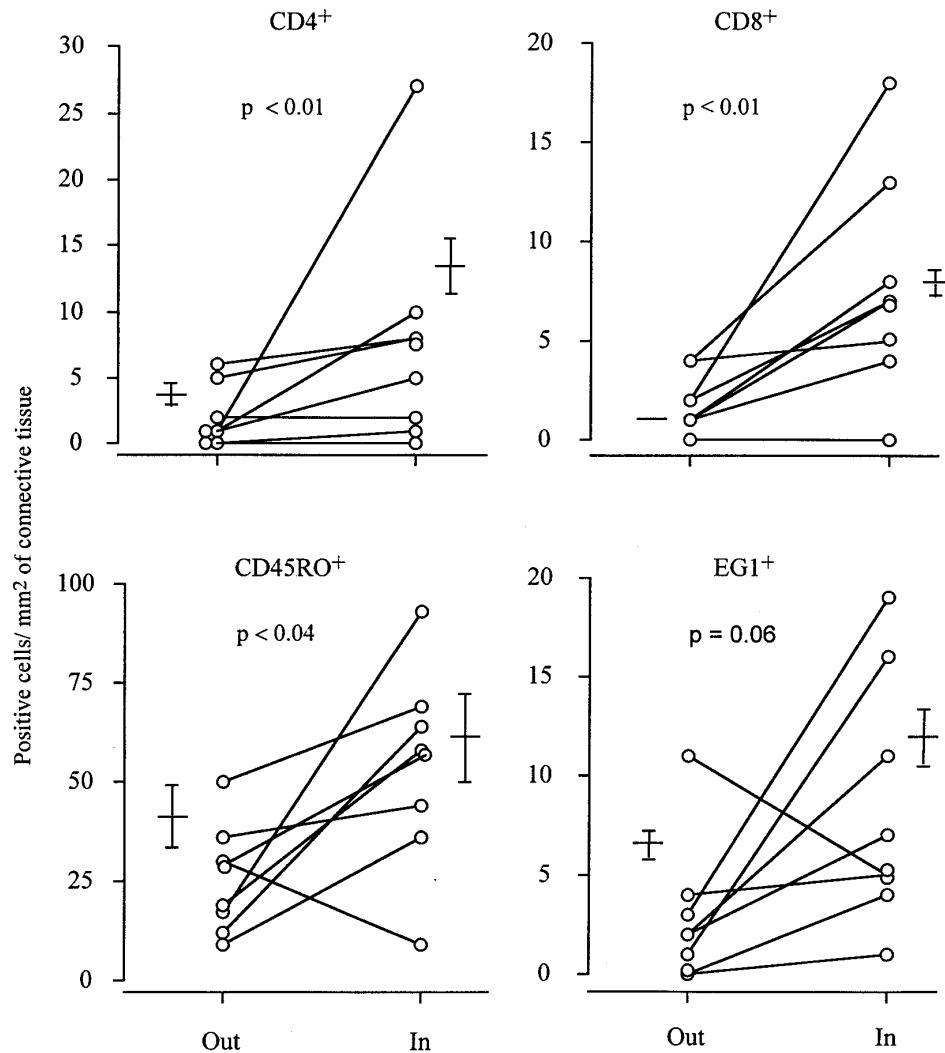


FIG 2. Seasonal comparison of CD4⁺, CD8⁺, CD45RO⁺, and EG1⁺ cell counts in the bronchial submucosa of nonasthmatic subjects with rhinitis. Immunostaining of bronchial biopsy specimens with a panel of antibodies shows that the number of CD4⁺, CD8⁺, and CD45RO⁺ cells significantly increased during the pollen season. There was also an increase in the number of eosinophils during the pollen season, although the changes did not reach statistical significance. Horizontal bars represent means $\pm$ SEM.

RESULTS

Change in airway responsiveness

We recruited 12 subjects: two did not agree to the second bronchoscopy, and bronchial biopsy specimens from two others were not adequate. The final study population consisted of 8 subjects (2 female and 6 male subjects). These subjects with rhinitis had normal pulmonary functions (Table I). The mean out-of-pollen-season PC₂₀ value was 64 ± 15 mg·mL⁻¹. This value decreased after natural pollen exposure (33 ± 8 mg·mL⁻¹, $P = .01$) without reaching the asthmatic range (>8 mg/mL, Fig 1).

Changes in bronchial mucosal inflammatory cells

The number of different lymphocyte subpopulations increased significantly during the pollen season: CD4⁺ cells,

4 ± 0.7 to 12 ± 6 cells/mm² ($P = .01$); CD8⁺ cells, 2 ± 0.4 to 8 ± 2 cells/mm² ($P = .01$); and CD45RO⁺ cells, 34 ± 4 to 55 ± 10 cells/mm² ($P = .04$). There was also an increase in the number of eosinophils during the pollen season, although the changes did not reach statistical significance (7 ± 1 to 12 ± 3 cells/mm², $P = .06$; Fig 2). There was no increase in mast cell counts (tryptase-positive cells, 32 ± 6 to 31 ± 7 cells/mm² [$P = .73$]; EG2⁺ cells, 8 ± 5 to 7 ± 2 cells/mm²; CD25⁺ cells, 10 ± 8 to 5 ± 2 cells/mm²; and HLA-Dr⁺ cells, 36 ± 7 to 42 ± 11 cells/mm²) during the pollen season.

There was a significant correlation between the numbers of CD45RO⁺ cells and the number of HLA-Dr⁺ cells during the pollen season ($r = 0.82$, $P < .02$). There was also a correlation between the number of EG2⁺ cells during the pollen season and the number of CD25⁺ cells ($r = 0.88$, $P < .003$).

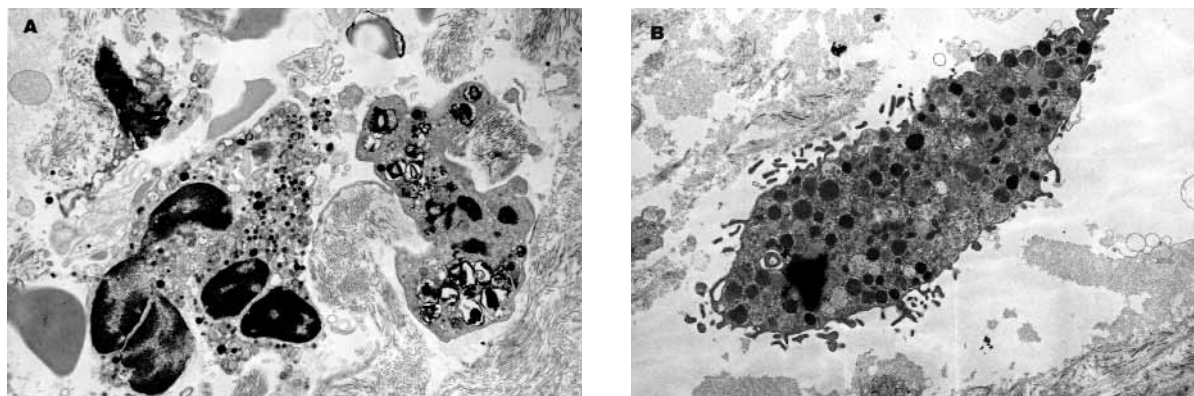


FIG 3. Electron microscopy micrographs of bronchial biopsy specimens obtained from nonasthmatic subjects with rhinitis. **A**, Degranulating eosinophil in a bronchial biopsy specimen from a nonasthmatic subject with rhinitis in the pollen season. **B**, Mast cell with cytoplasmic granules obtained from a bronchial biopsy specimen of a nonasthmatic subject with rhinitis in the pollen season.

Electron microscopy analysis of bronchial biopsy specimens during the pollen season showed degranulated eosinophils and undegranulated mast cells (Fig 3, A and B).

There was no correlation between changes in inflammatory cell counts and changes in methacholine PC₂₀.

Changes in cytokine expression

Bronchial biopsy specimens taken after natural exposure to pollen showed an increased immunoreactivity for IL-5 (75.7 ± 6.4 cells/mm², $P = .01$) compared with out-of-season biopsy specimens (35.7 ± 9.5 cells/mm²; Fig 4, A). Fig 4, B, shows a photomicrograph displaying IL-5 immunoreactivity in and out of the pollen season in the same subject. The numbers of IL-4⁺ cells (15 ± 4 cells/mm² in season, 10 ± 3 cells/mm² out of season) and IFN- γ ⁺ cells (19 ± 4 cells/mm² in season, 21 ± 7 cells/mm² out of season) remained unchanged after natural exposure.

The increase in IL-5 expression correlated with the numbers of CD3⁺ cells ($r = 0.96$, $P = .018$), CD4⁺ cells ($r = 0.96$, $P = .018$), CD45RO⁺ cells ($r = 0.86$, $P = .035$), and EG1⁺ cells ($r = 0.86$, $P = .035$; Fig 5). There was no correlation between the increase in IL-5 and CD8⁺ cells.

DISCUSSION

This study showed that there is an inflammatory response in the bronchial mucosa of nonasthmatic subjects with allergic rhinitis and suggests that this inflammation is increased after natural exposure to pollen. This inflammatory burst is characterized by a significant increase in the number of CD4⁺ and CD8⁺ lymphocytes, memory cells, and eosinophils, as well as increased IL-5 expression. The number of IL-5⁺ cells was correlated with the numbers of CD3⁺, CD4⁺, CD45RO⁺, and EG1⁺ cells, suggesting that lymphocytes and eosinophils may be the main source of this cytokine in the bronchial mucosa of nonasthmatic subjects with rhinitis. Consequently, the release of IL-5 in the bronchial mucosa of subjects with rhinitis may contribute, at least in part, to the increase in eosinophil cell numbers.

The upregulation of the inflammatory response likely triggered by exposure to allergens, particularly the accumulation of eosinophils and lymphocytes in the bronchial mucosa of subjects with rhinitis, could contribute to the mild form of airway remodeling observed in these subjects. We have previously reported that there is lower airway inflammation documented by histology¹³ and a focal deposition of type I and III collagen under the basement membrane of subjects with rhinitis. The deposition falls between that found in normal and asthmatic airways.¹⁶ Taken together, these two findings suggest that the bronchial mucosa of subjects with rhinitis is the active site of both inflammation and remodeling after seasonal exposure to pollen.

Interestingly, the numbers of IFN- γ ⁺ and IL-4⁺ cells did not rise significantly in airway bronchial biopsy specimens from subjects with rhinitis after natural pollen exposure. This suggests the absence of a predominant lower airway T_H2 response in nonasthmatic subjects with rhinitis compared with asthmatic subjects. Djukanovic et al²² reported that the natural exposure of asthmatic subjects with seasonal rhinitis results in increased IL-4 secretion in the bronchial mucosa. This may distinguish between the physiologic response of nonasthmatic and asthmatic subjects with rhinitis that occurs during the pollen season.

It would be interesting to compare inflammatory changes in both the nasal and bronchial mucosa after natural seasonal exposure. In fact, Bentley et al²³ have shown that seasonal exposure increases mast cell and eosinophil counts in the nasal mucosa of subjects with allergic rhinitis without changing T-lymphocyte counts. The most important difference we found between the nasal mucosa of subjects evaluated by Bentley et al and the bronchial mucosa in our subjects after natural exposure was the significant increase in T lymphocytes, specifically in memory T cells. We also found no changes in the cell counts of tryptase-positive cells, and electron microscopic analysis revealed that these cells were not degranulated. Our observations would be strengthened if both nasal and bronchial biopsy specimens were taken in the same subjects. These

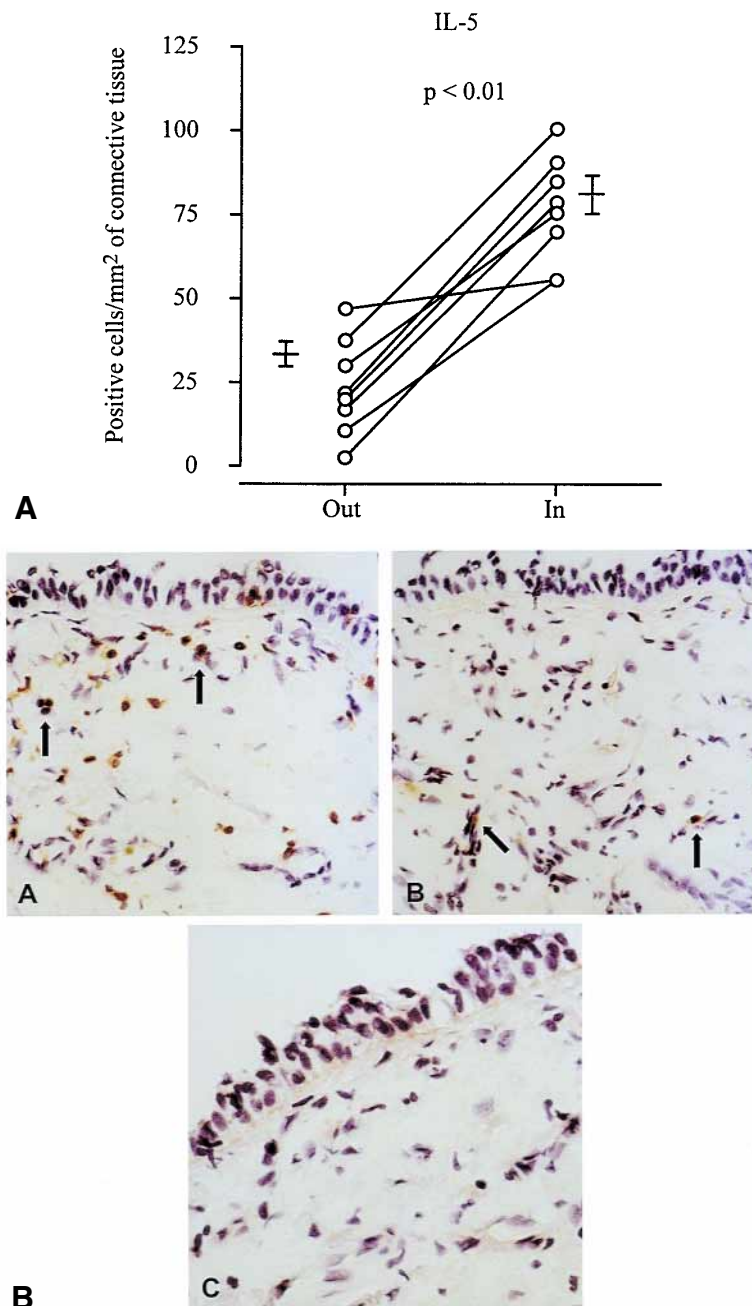


FIG 4. A, IL-5⁺ cell counts in bronchial submucosa of nonasthmatic subjects with rhinitis. IL-5⁺ cell counts increased significantly in the pollen season compared with the out-of-season period ($P = .01$). B, Photomicrograph showing IL-5 immunoreactivity. Arrows indicate immunostained cells in (A) and out (B) of the pollen season. C represents a negative control (magnification 200 $\times$).

observations suggested that the immune response in bronchial mucosa compared with that in the nasal mucosa solicited different immune cells, T cells in the first case and mast cells in the second. This may be explained by a tissue-specific response or by the nature of the aeroallergens that reach the bronchial mucosa by oral breathing.

Natural allergen exposure is associated with an increase in airway responsiveness in our subjects with-

out, however, reaching the asthmatic range. These changes in airway hyperresponsiveness may be a consequence of lower airway inflammation, although we found no correlation between changes in inflammatory cell counts and changes in methacholine PC₂₀ values. The inflammatory response observed in the lower airways of our subjects was similar to that observed in asthmatic subjects. In stable asymptomatic asthma, baseline

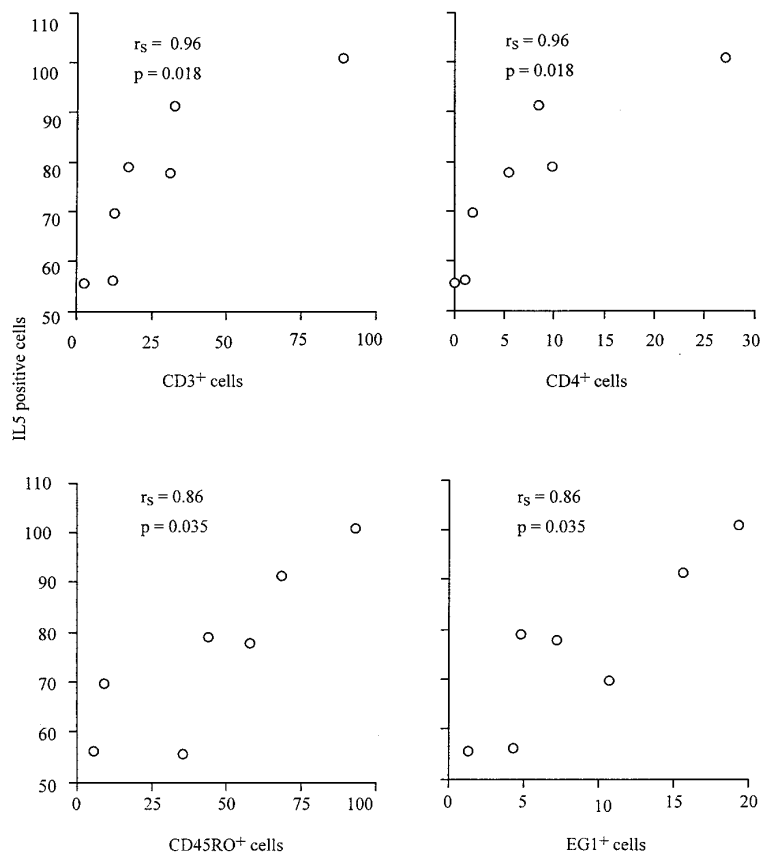


FIG 5. Correlation of CD3⁺, CD4⁺, CD45RO⁺, and EG1⁺ cell counts with IL-5⁺ cell counts in bronchial submucosa of nonasthmatic subjects with rhinitis. The increase in IL-5⁺ cell counts during the pollen season correlated with the number of CD3⁺, CD4⁺, CD45RO⁺, and EG1⁺ cells (Spearman rank correlation test).

TABLE I. Subject characteristics

	Sex	Age (y)	FEV ₁ (% predicted)	FVC (% predicted)
1	F	22	109	113
2	M	21	142	136
3	M	35	116	109
4	M	27	90	95
5	M	20	117	113
6	F	31	106	103
7	M	23	111	103
8	M	35	104	99
Mean ± SEM	—	27 ± 2	112 ± 5	108 ± 5

FVC, Forced vital capacity.

airway responsiveness did not correlate with the presence of inflammatory cells recovered by bronchoalveolar lavage (BAL) in the airways, whereas allergen-induced increases in airway responsiveness were associated with an influx of eosinophils and lymphocytes into the bronchial lumen.²⁴

To determine why nonasthmatic patients with allergic rhinitis differ in their airway response to inhaled allergens compared with patients with allergic asthma, Lam et al²⁵ analyzed the BAL fluid of 10 subjects with aller-

gic asthma and 5 with allergic rhinitis before and after allergen inhalation challenge. After the challenge, the asthmatic subjects released significant amounts of histamine and thromboxane B₂ into the BAL fluid, whereas subjects with rhinitis alone did not. After allergen challenge, there was also a significant increase in PGE₂ levels in the asthmatic subjects. Lam et al suggested that subjects with allergic asthma differ from subjects with rhinitis alone in the capacity to release more mediators into the airways on allergen challenge.

In conclusion, we found that the bronchial mucosa of nonasthmatic subjects with allergic rhinitis displayed the features of an active inflammatory response characterized by increased IL-5 immunoreactivity and recruitment of inflammatory cells, such as lymphocytes and eosinophils. This inflammatory process increased in association with natural allergen exposure and might constitute an increased risk for development of symptomatic asthma in predisposed individuals. Further studies will be necessary to evaluate the clinical and physiologic consequences of this bronchial inflammation in nonasthmatic subjects with rhinitis.

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