

Relationship between airway inflammation, hyperresponsiveness, and obstruction in asthma

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Background: Although the role of eosinophils in airway inflammation in chronic asthma has been extensively studied, a role for neutrophils has not been well characterized. Furthermore, prior studies have not systematically sought or controlled for factors that might confound the relationship between cellular markers of inflammation and physiologic measures of airway function.

Objective: The purpose of this study was to determine whether eosinophilic and neutrophilic inflammation independently contribute to abnormalities of airway function in asthma.

Methods: Multivariate analysis of data collected during screening and enrollment of 205 asthmatic adults for clinical trials was conducted to examine the relationships between cellular inflammation in induced sputum and FEV₁ and methacholine responsiveness (PC₂₀) while confounding factors were controlled for.

Results: We found that age, sex, ethnicity, and use of inhaled corticosteroids were important confounding factors of the relationship between cellular inflammation and airway function. When these factors were controlled for, multivariate analysis showed that eosinophil percentage in induced sputum is independently associated with lower FEV₁ and lower PC₂₀ ($P = .005$ and $P = .005$, respectively). In the same models, increased sputum neutrophil percentage is independently associated with lower FEV₁ ($P = .038$) but not with PC₂₀ ($P = .49$).

Conclusions: These results suggest that both eosinophilic inflammation and neutrophilic inflammation independently contribute to abnormalities of FEV₁ in asthma. Therapies directed specifically at control of neutrophilic inflammation might be useful in improving airway caliber in patients with chronic asthma. (*J Allergy Clin Immunol* 2001;108:753-8.)

Key words: Asthma, airway obstruction, eosinophils, neutrophils

Despite many studies of airway inflammation in asthmatic subjects in the past 20 years,¹⁻³ the relationships between cellular markers of airway inflammation and measures of airway function, such as FEV₁ and methacholine responsiveness (provocative concentration of methacholine that leads to a 20% drop in FEV₁ [PC₂₀]), remain unclear. There are at least 2 reasons for this. First, studies that have examined these relationships have been relatively small and so have had limited power. Second, these studies have not systematically controlled for covariates such as demographic factors and medication use, which might confound the relationship between cellular markers of inflammation and physiologic measures of airway function.

Sputum induction is a noninvasive method of collecting airway secretions and has been validated as a method for assessing airway inflammation in asthma.^{4,5} Induced sputum represents a more concentrated sample of airway secretions than bronchoalveolar lavage and is derived more from the airway compartment of the lung than from the alveolar compartment.⁶ Sputum induction is now performed routinely in clinical research protocols in asthmatic subjects, and we have developed a database of information regarding asthmatic subjects that includes measures of cellular inflammation in induced sputum, FEV₁, and PC₂₀.

We hypothesized that abnormalities in FEV₁ and PC₂₀ might be associated with inflammatory cells in sputum, such as the eosinophil and the neutrophil. We performed a retrospective review of our asthma subject database to examine relationships between sputum markers of airway inflammation and FEV₁ and PC₂₀. We anticipated that several covariates, including age, sex, ethnicity, and use of inhaled corticosteroids, might confound these associations. Accordingly, our data analysis plan was designed to systematically evaluate relationships among these covariates, sputum markers of inflammation, and airway function and to use multivariate linear regression analysis to control for confounding effects.

METHODS

Subjects

Data had been entered into a database (Filemaker Pro 3.0, Filemaker Inc, Santa Clara, Calif) on 205 nonsmoking subjects with stable asthma who had undergone standardized 12-minute sputum induction, spirometry, and methacholine challenge during enroll-

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Abbreviations used

PC₂₀: Provocative concentration of methacholine that leads to a 20% drop in FEV₁

IQR: Interquartile range

ment in outpatient asthma studies performed during the past 5 years at the Asthma Research Center at the University of California, San Francisco. The subject population comprised all individuals with asthma who participated in outpatient clinical studies during this period and who underwent sputum induction, spirometry, and methacholine challenge. Each of the subjects had a prior diagnosis of asthma and symptoms of asthma (episodic shortness of breath, wheezing, or cough) at evaluation during the study. For every subject, the clinical diagnosis of asthma was confirmed by methacholine challenge (PC₂₀ < 8 mg/mL). Exclusion criteria included a history of smoking more than 10 pack-years, any history of smoking in the past year, or a history of upper respiratory tract infection in the last 6 weeks. The local institutional review board approved all of the studies in which these subjects were initially enrolled, and written informed consent for participation in the original studies was obtained from every subject.

Measurements

After an 8-hour bronchodilator hold, spirometry was performed through use of one of 3 spirometers, depending on the original research study in which the subject had been enrolled (Ohio 840 rolling seal spirometer, Medtronic Sales, Inc, Burbank, Calif; Survey spirometer [catalog no. 06036], Warren E. Collins, Inc, Braintree, Mass; Survey II spirometer [catalog no. 06047], Warren E. Collins, Inc). All spirometric measurements were performed according to the American Thoracic Society criteria.⁷ Predicted values for spirometric measurements used in this study were generated from the same set of regression equations for all subjects⁸ to standardize calculation of percentage of predicted FEV₁. Methacholine challenge was performed through use of a method modified from Chai et al.⁹

Sputum induction was performed according to a standardized 12-minute protocol through use of inhaled nebulized sterile 3% saline solution.¹⁰ Subjects were instructed to spit saliva into one plastic container before coughing sputum into another. Sputum volume was determined and samples were processed with 0.1% dithiothreitol (Sputalysin, Behring Diagnostics, Inc, Somerville, NJ) in saline solution, as previously described.⁴ Samples with >80% squamous cells were considered inadequate and not included in this analysis. Squamous cells were excluded from the calculation of cell differentials; each cell concentration is expressed as the number of cells per milliliter of sputum.

Statistical considerations

Data analysis was performed through use of STATA version 5.0 (STATA Corp, College Station, Tex). Initial univariate analyses were performed to assess relationships between demographic characteristics, use of inhaled corticosteroids, and sputum indices of inflammation (percentages of neutrophils and eosinophils) through use of *t* testing, Wilcoxon rank sum testing, and Spearman correlation where appropriate. Similarly, univariate analyses of the relationship between these variables and the outcomes of interest (FEV₁ and PC₂₀) were performed. Multivariate linear regression models were developed to assess the relationship of eosinophil percentage and neutrophil percentage to the outcomes of interest while controlling for the predictor variables that were found to be important as potential confounders of the analysis. Analyses were repeated

through use of eosinophil and neutrophil concentrations (number of cells per milliliter of sputum). Regression diagnostics were performed, and the adequacy of covariate functional forms was examined. Summaries include regression coefficients, SEs, and *P* values corresponding to 2-sided testing of zero regression effects.

RESULTS**Patient characteristics**

A total of 205 subjects for whom spirometry, methacholine challenge, and sputum data were available were identified (Table I). A small majority (56%) were female, and approximately one third overall (32%) were of non-white ethnicity. Almost one half of the subjects demonstrated an FEV₁ no greater than 80% of predicted, and 40% of subjects were using inhaled corticosteroids. The median volume of induced sputum obtained was 4.6 mL (interquartile range [IQR], 2.7, 7.0). The predominant nonsquamous cell types in our subjects' induced sputum were macrophages and neutrophils, which together constituted 80% of the nonsquamous cells counted (Table II).

Relationships between covariates

Initial univariate analyses revealed important relationships between certain covariates. Older age correlated with increased sputum neutrophil percentage ($r = 0.27$; $P < .001$ by Spearman correlation) but not eosinophil percentage ($r = 0.05$; $P = .48$; Fig 1), and older age also correlated with lower FEV₁, even when FEV₁ was expressed as a percentage of predicted value ($r = -0.27$; $P < .001$). Because an analysis that did not adjust for age might overestimate the association between sputum neutrophilia and FEV₁, we controlled for age in multivariate linear regression modeling.

There was statistically significant variation in sputum neutrophilia by ethnicity ($P = .05$ by ANOVA), the highest levels being seen in African American subjects. The percentage of sputum eosinophils was lowest in African American subjects, with a trend toward variation across the 4 ethnic groups ($P = .07$ by ANOVA). Because of these relationships, ethnicity was included as a covariate in multivariate modeling. There was a trend toward higher sputum eosinophil percentage in female subjects than in male subjects (7.2% vs 4.4%; $P = .06$ by Wilcoxon test). Furthermore, there was a trend toward lower FEV₁ (even when corrected for sex) among the men in our group of asthmatic subjects (80.5% ± 16.4% vs 84.3% ± 16.2%; $P = .10$). For these reasons, and because of its potential importance as a basic demographic variable, sex was included in multivariate modeling.

Inhaled corticosteroid use demonstrated different effects on sputum eosinophilia and neutrophilia (Fig 2). Use of inhaled corticosteroids was associated with lower eosinophil percentage (median, 1.6% [IQR, 0.3% to 5.9%] vs 3.1% [IQR, 0.8% to 9.7%]; $P = .02$ by Wilcoxon test). However, there was no association between use of inhaled corticosteroids and sputum neutrophil percentage (40.9% [IQR, 22.8% to 59.4] vs 35.4% [IQR, 24.0% to 51.9%] among users of inhaled corticosteroids and nonusers respectively; $P = .48$). Of interest, cortico-

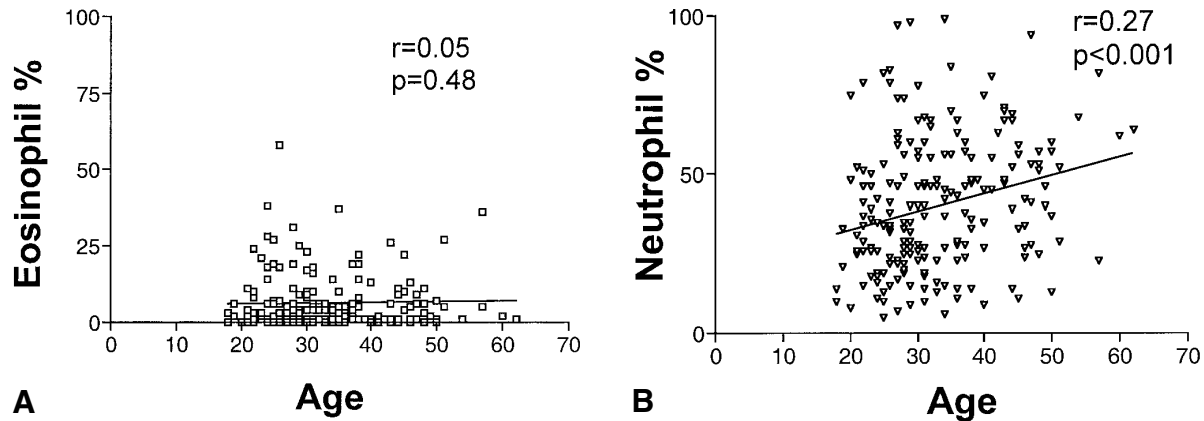


FIG 1. Relationship of age to induced sputum eosinophil percentage (A) and sputum neutrophil percentage (B) in 205 subjects with asthma.

steroid use varied by age; subjects using inhaled corticosteroids were older (35.6 ± 9.8 years vs 31.3 ± 8.1 years; $P < .001$ by t test) than subjects not using inhaled corticosteroids, and the analyses discussed above demonstrate that age relates to FEV_1 . To adjust for potential confounding on the basis of corticosteroid use, we controlled for use of inhaled corticosteroid use in the multivariate analysis.

With regard to cellular components of sputum, neutrophil percentage showed strong negative correlation with macrophage percentage ($r = -0.78$; $P < .001$ by Spearman correlation). Because macrophages and neutrophils together account for the vast majority of non-squamous cells in induced sputum from normal subjects¹¹ as well as from the asthmatic subjects in our study, we interpreted strong negative correlation as indicating that an increase in percentage of one cell type necessitates a decrease in percentage of the other. We therefore treated these covariates as strongly collinear and excluded macrophage percentage from multivariate analyses that included neutrophil percentage.

Unadjusted analyses

In unadjusted analyses, higher percentages of eosinophils in induced sputum were associated with lower PC_{20} ($r = -0.26$; $P < .001$ by Spearman correlation), and there was a trend toward association with lower FEV_1 ($r = -0.13$; $P = .06$). Higher neutrophil percentage in induced sputum, on the other hand, was not associated with PC_{20} ($r = -0.04$; $P = .52$) but was associated with lower FEV_1 ($r = -0.15$; $P = .03$).

Multivariate linear regression analyses

Multivariate linear regression models were developed for both outcomes of interest (percentage of predicted FEV_1 as a measure of chronic airway obstruction and PC_{20} to methacholine as a measure of airway hyperresponsiveness). The values for PC_{20} were log-transformed to conform with model assumptions. All models contain the following covariates: age, sex, ethnicity (4-category variable), inhaled corticosteroid use (dichotomous vari-

TABLE I. Characteristics of the 205 subjects with asthma

Characteristic	Value*
Age (y): mean $\pm$ SD	33.0 $\pm$ 9.1
Sex: female	114 (56%)
Ethnicity	
White	140 (68%)
African American	25 (12%)
Hispanic	18 (9%)
Other	22 (11%)
On inhaled corticosteroids	83 (40%)
Percent of predicted FEV_1	
<60%	17 (8%)
61% to 80%	76 (37%)
>80%	112 (55%)
PC_{20} : median (interquartile range)	0.41 (0.18, 1.05)

*Presented as number (percent) unless otherwise indicated.

TABLE II. Nonsquamous cellular constituents of sputum induced from 205 subjects with chronic asthma

Cell type	Percent (mean $\pm$ SD)
Macrophages	40.1 $\pm$ 19.0
Neutrophils	40.0 $\pm$ 21.0
Epithelial cells	12.6 $\pm$ 11.5
Eosinophils	6.0 $\pm$ 8.6
Lymphocytes	1.3 $\pm$ 1.3

able), percentage eosinophils, and percentage neutrophils (Table III). When FEV_1 was considered the outcome of interest, demographic factors associated with lower FEV_1 included older age and male sex. Higher sputum eosinophil and neutrophil percentages were independently associated with lower FEV_1 when we controlled for the other covariates and for each percentage. However, the association between FEV_1 and eosinophil percentage was relatively stronger than the relationship between neutrophil percentage and FEV_1 . When methacholine PC_{20} was considered the outcome of interest, only sputum eosinophil percentage was associated with lower PC_{20} .

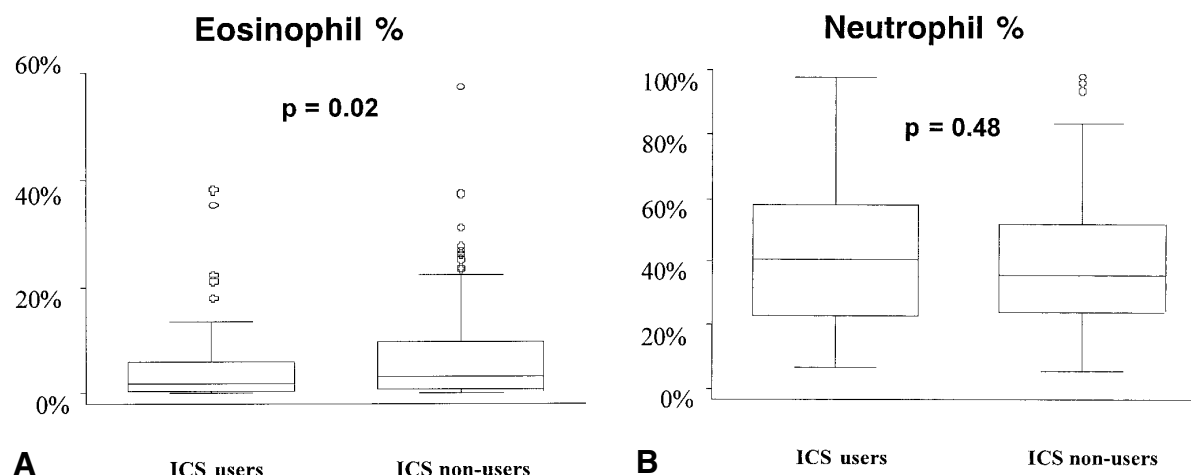


FIG 2. Relationship of inhaled corticosteroid use to sputum eosinophil percentage (**A**) and sputum neutrophil percentage (**B**). *ICS*, Inhaled corticosteroids.

TABLE III. Results of multivariate linear regression modeling of the relationship of sputum percentage of eosinophils and neutrophils to FEV₁ and PC₂₀

Outcome	Variable	Coefficient	SE	P value
FEV ₁	Neutrophil percentage	−0.11	0.05	.038
	Eosinophil percentage	−0.38	0.13	.005
PC ₂₀ (log-transformed)	Neutrophil percentage	−0.001	0.002	.49
	Eosinophil percentage	−0.01	0.005	.005

Age, sex, ethnicity, and inhaled corticosteroid use were controlled for.

Modeling through use of concentrations of cells

When analyses were performed through use of concentrations of eosinophils and neutrophils (numbers per milliliter of sputum) rather than percentages, the results differed. In unadjusted analyses, higher eosinophil concentration was associated both with lower PC₂₀ ($r = -0.21$; $P = .003$ by Spearman correlation) and with lower FEV₁ ($r = -0.15$; $P = .03$). Higher neutrophil concentration was not associated with PC₂₀ ($r = 0.004$; $P = .95$) but showed a trend toward association with lower FEV₁ ($r = -0.13$; $P = .07$). Multivariate linear regression models were developed that controlled for all of the covariates included in previous models as well as for the number of nonsquamous cells per milliliter. This additional adjustment was performed because the number of nonsquamous cells per milliliter was found to be a confounder of the relationship between eosinophil and neutrophil concentration and FEV₁ in multivariate modeling. These findings indicate that variable contamination with squamous cells between samples might influence the measured concentrations of eosinophils and neutrophils. In these models, higher eosinophil concentration was associated with lower FEV₁ ($P = .02$) but not with PC₂₀ ($P = .31$). In addition, higher neutrophil concentration was associated with lower FEV₁ at a borderline level of statistical significance ($P = .07$) but was not associated with PC₂₀ ($P = .28$).

Other medication use

Only 11 of the 205 subjects in this study were using long-acting β -agonists. However, because there is emerging evidence that long-acting β -agonist use might have anti-inflammatory effects or might potentiate the anti-inflammatory effects of corticosteroids, we performed further multivariate regression modeling to control for any effects. We found that controlling for the use of long-acting β -agonists did not materially change the results of any of the models (data not shown). Thus there is no discernable effect of long-acting β -agonist use on the relationships between cellular inflammation and lung function in this study.

DISCUSSION

Our multivariate models suggest that both eosinophilic inflammation and neutrophilic inflammation in sputum are independently associated with degree of chronic airway obstruction, as measured by FEV₁. With regard to bronchial hyperreactivity, sputum eosinophilia shows a relationship with PC₂₀ whereas sputum neutrophilia does not. The finding that sputum neutrophilia relates to FEV₁ but not to PC₂₀ was surprising. Although the mechanisms governing chronic airway narrowing and chronic airway hyperreactivity in asthma overlap, there are likely to be several distinct structural and functional changes that

mediate these abnormalities. Our data suggest that the neutrophil might be an important mediator of airway caliber in asthma but might not be an important mediator of bronchial hyperreactivity.

Our analyses used a multivariate approach that controlled for demographic characteristics and medication use. Adjustment for these factors is important; our preliminary analyses revealed that age, sex, ethnicity, and use of inhaled corticosteroids might influence sputum indices of inflammation, airway function, or both. Consequently, unadjusted analyses might produce artifactual results.

Although eosinophilic inflammation has long been considered a hallmark of asthma,^{1,12} neutrophilic inflammation has only recently been considered a potential contributor. Several studies have demonstrated neutrophilic inflammation in the airway during exacerbations of asthma and with status asthmaticus.¹³⁻¹⁶ Initial evidence for neutrophilic inflammation in chronic asthma rather than acute exacerbations was provided by Wenzel et al,¹⁷ who demonstrated increased neutrophils in endobronchial biopsy specimens in a study of asthmatic individuals with severe corticosteroid-resistant chronic asthma.

A pair of more recent studies has suggested a relationship between neutrophilic inflammation in induced sputum and severity of chronic asthma,^{18,19} but neither study demonstrated a relationship between neutrophils and FEV₁ and neither sought or adjusted for potential confounders. Jatakanon et al¹⁸ compared induced sputum profiles in 12 healthy control subjects and 55 patients classified into 3 groups on the basis of severity of asthma according to clinical criteria. Sputum examination demonstrated increased neutrophilia in subjects with severe asthma in comparison with subjects with mild asthma or healthy controls. However, corticosteroid use differed systematically between the groups, leaving open the possibility that the finding was an effect of differential treatment. Similarly, Louis et al¹⁹ examined the induced sputum of 22 nonatopic control subjects and 74 asthmatic subjects classified into 3 severity groups on the basis of clinical criteria. Sputum neutrophils were significantly increased in those with severe asthma in comparison with those with intermittent asthma, and increased sputum neutrophils were associated with peak flow rate variability (but not FEV₁). The authors performed subgroup analyses to assess the effects of corticosteroids on inflammatory cell numbers and found contradictory effects of high-dose inhaled corticosteroids in comparison with oral corticosteroids (which were associated with increased sputum neutrophil counts and decreased sputum neutrophil counts, respectively). Consequently, it was difficult to assess the effect of corticosteroid use on their analyses. Neither study considered the potential roles of age, sex, and ethnicity as potential confounders.

Neutrophils and their products could contribute to airway narrowing in asthma in several ways. Neutrophils might cause airway narrowing secondary to mucus hypersecretion either indirectly, through the production of mediators such as neutrophil elastase, or directly, through direct goblet cell/neutrophil interactions.²⁰⁻²² In

addition, neutrophil products might be important mediators of epithelial cell activation.²³ Finally, neutrophil products can activate eosinophils.²⁴ That neutrophils might exert specific structural effects on the airway, leading to chronic airway narrowing as a downstream effect of tissue injury or through the release of pro-inflammatory or profibrotic cytokines, is less clear.

The strength of the relationships between airway function measures and sputum leukocytes was stronger when the sputum leukocytes were expressed as percentages than when they were expressed as concentrations. For example, for FEV₁, multivariate regression modeling revealed a significant association with sputum eosinophils expressed either as a percentage or as a concentration, but the relationship was stronger for eosinophils expressed as a percentage. Similarly, the relationship between FEV₁ and sputum neutrophils was stronger when the neutrophils were expressed as a percentage. For PC₂₀, multivariate regression modeling revealed a significant association with sputum eosinophils, but only when eosinophils were expressed as a percentage; there was no significant association between PC₂₀ and sputum eosinophil concentration. In this study, we chose *a priori* to use sputum neutrophils and eosinophils expressed as the percentage of nonsquamous cells as the primary outcomes of airway inflammation. Sputum cell percentages, not sputum cell numbers, are the outcomes of choice in our laboratory because the use of percentages internally controls for the variable dilution of sputum by saliva and because sputum cell concentrations have more measurement variability.⁴ For example, the reproducibility of sputum eosinophil and neutrophil percentages is good^{25,26} and has been shown to be superior to that of total cell counts.²⁷ Accordingly, our approach to the inconsistency in findings for the relationship between PC₂₀ and sputum eosinophils is to assign more weight to the association found when sputum eosinophils are expressed as a percentage.

An important feature of our study is that we systematically examined and controlled for demographic factors that might confound the relationship between sputum cellularity and FEV₁. To our knowledge, systematic examination of potential confounders has not been performed previously in studies relating airway inflammation and asthma severity. We found that percentage of predicted FEV₁ decreases with age, which is consistent with findings in a recent study demonstrating accelerated decline in pulmonary function in asthma.²⁸ When combined with our finding that sputum neutrophilia, but not eosinophilia, increases with age, these relationships identify age as a potential confounder of our analysis. We also found that inhaled corticosteroid use was associated with lower sputum eosinophilia but not with neutrophilia. This suggests that airway neutrophils might be resistant to the effects of inhaled corticosteroids, and it raises the possibility that a relative increase in the percentage of sputum neutrophils in severe asthma could be secondary to a treatment effect. Consequently, we controlled for use of inhaled corticosteroids in our analysis.

The present study has certain potential limitations. Because data were collected in independent clinical studies, some aspects of the protocol varied over time. For example, spirometry was performed with 3 different instruments over this period. However, all of the instruments meet American Thoracic Society standards, and the percentage-of-predicted values were recalculated through use of updated regression equations to maximize standardization for this analysis.⁸ A specific advantage of these regression equations is that they were developed from a sample of the US population that included African American and Mexican American ethnic groups to produce specific equations for each ethnic group rather than post hoc corrections. We must also emphasize that these subjects do not represent a population sample and might not represent the most severely affected spectrum of patients with asthma (all subjects were well enough to undergo methacholine challenge testing). Nonetheless, these subjects are representative of a relatively broad range of patients with mild to moderate asthma.

In summary, cellular inflammation in induced sputum might vary by age, sex, and ethnicity and might be influenced by the use of inhaled corticosteroids. In a multivariate analysis that adjusts for these factors, eosinophilic inflammation is independently associated with degree of chronic airway obstruction as measured by FEV₁ and degree of bronchial hyperreactivity as measured by PC₂₀. Increased neutrophilic inflammation, on the other hand, is associated with decreased FEV₁ but not PC₂₀. Because sputum neutrophilia was not decreased in subjects using inhaled corticosteroids, other anti-inflammatory therapies directed specifically at control of neutrophilic inflammation might be useful in improving airway caliber in patients with more severe disease.

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