

Eosinophils, mast cells, and basophils in induced sputum from patients with seasonal allergic rhinitis and perennial asthma: Relationship to methacholine responsiveness

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Objectives: We attempted to determine whether inflammation is present in induced sputum of patients with seasonal allergic rhinitis (AR) as compared with those with perennial asthma (AS) and examined its relationship with bronchial responsiveness to methacholine.

Methods: Sputum was induced in 30 patients with seasonal rhinitis in response to grass pollens only and in 15 patients with stable, asymptomatic asthma. The AR group was divided according to methacholine PD₂₀ value: the AR- group ($n = 15$) had a methacholine PD₂₀ greater than 24 μmol ; the AR+ group ($n = 15$) had a methacholine PD₂₀ ranging between 2.2 and 19.6 μmol . In the AS group, methacholine PD₂₀ ranged between 0.42 and 2.6 μmol . The percentage of eosinophils and metachromatic cells (alcian blue-positive) was assessed in sputum by light microscopy. Tryptase-positive cells and EG2+ cells were identified by immunocytochemistry with the mouse anti-human mast cell-tryptase monoclonal antibody and the monoclonal anti-eosinophil cationic protein antibody.

Results: We found that the number of eosinophils in the AS group was greater than that in the AR+ group ($p < 0.05$) and in the AR- group ($p < 0.01$). Moreover, the eosinophil count was lower in the AR- group compared with the AR+ group ($p < 0.05$). Similarly, the number of EG2+ cells was greater in the AS group than in the AR group ($p < 0.02$) and the AR- group ($p < 0.05$). Moreover, the EG2+ cell count was lower in the AR- group than in the AR+ group ($p < 0.05$). The number of mast cells and basophils in the AS group was greater than that in the AR group ($p < 0.05$ and $p < 0.01$, respectively). Mast cells in sputum were tryptase-positive. Basophils were present in sputum from 23% of patients with AR and 53% of patients with asthma. There was a significant correlation between methacholine PD₂₀ and eosinophils ($p < 0.005$) and mast cells ($p < 0.02$) but not with basophils in those patients showing a measurable methacholine PD₂₀ (AR+ and AS groups).

Conclusions: Inflammatory cells are present not only in the airways of patients with asthma but also in airways of patients with seasonal AR, even outside natural exposure.

Moreover, we provide evidence for the presence of basophils in sputum of patients with asthma even during clinical remission. The presence of bronchial responsiveness is associated with an increase in the number of eosinophils and metachromatic cells. Our findings are consistent with the hypothesis that eosinophils, as well as mast cells, contribute to bronchial responsiveness not only in AS but also in seasonal AR. (*J Allergy Clin Immunol* 1997;100:58-64.)

Key words: Seasonal allergic rhinitis, allergic asthma, airway inflammation, sputum, mast cells, basophils, eosinophils

Chronic bronchial inflammation is a major determinant in the pathogenesis of asthma.¹ It is present even in patients with mild disease² and increases during natural exposure³ or provoked exacerbation.⁴ Seasonal allergic rhinitis (AR) is a disease that often precedes the onset of clinical asthma and could represent a predisposing condition or an early stage in the development of asthma.⁵ The presence of an inflammatory process in the lower airways of patients with seasonal AR has also been suggested by a few studies.⁶⁻⁹

The analysis of cells in bronchoalveolar lavage (BAL) and biopsy specimens has demonstrated that the inflammatory infiltrate in asthma mainly consists of eosinophils and metachromatic cells.^{1,4} More recently, induced sputum has been proposed and validated as a noninvasive alternative method with which to assess airway inflammation.^{10,11} Cytologic analysis of induced sputum has confirmed these findings.¹⁰⁻¹² Metachromatic cells likely sustain airway inflammation in asthma by a continuous release of mediators.^{13,14} Mast cells are almost exclusively located in connective tissues, whereas basophils are circulating granulocytes that migrate and accumulate at the inflammatory sites. Mast cells have central nuclei and peripheral granular cytoplasm. Their size varies between 5 to 7 and 12 to 18 μm . Mast cells contain tryptase as the predominant cytoplasmic protease. Basophils have multilobated nuclei, they contain a few granules, and their size varies between 5 and 6.5 μm . The contribution of basophils to the pathogenesis of airway inflammation has never been closely examined. However, it has recently been reported that basophils are increased in the airways of patients dying of asthma,¹⁵ during asthmatic exacerbation,¹⁶ and during the late asthmatic response to allergen.^{17,18} Basophils seem

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Received for publication July 1, 1996; revised Oct. 29, 1996; accepted for publication Feb. 6, 1997.

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0091-6749/97 \$5.00 + 0 1/181444

Abbreviations used

AR:	Allergic rhinitis
AS:	Perennial asthma
BAL:	Bronchoalveolar lavage

to be detectable in BAL and induced sputum from patients with asthma after staining with toluidine blue only occasionally.^{10, 19} However, counts of mast cells and basophils could likely be different if a different, more appropriate stain were applied. Previous studies in small groups of patients with seasonal AR demonstrate that eosinophils are present in the lower airways and that their absolute number in BAL⁸ and biopsy specimens⁹ is lower than that in patients with asthma. Interestingly, in patients with seasonal AR, the eosinophil count increases markedly after segmental allergen challenge²⁰ but not after natural exposure.²¹ However, cytologic analysis of induced sputum in seasonal AR has never been examined.

In asthma, the interrelationship between airway inflammation and bronchial responsiveness is a well established concept.²² However, very little is known about the pathogenesis of bronchial responsiveness associated with seasonal AR. It has been suggested that bronchial responsiveness in AR is caused by postnasal drip of mediators, which directly alters bronchial smooth muscle contractility²¹ rather than airway inflammation.

Therefore this study was designed with three objectives: (1) to determine whether inflammatory cells are present in induced sputum from patients with AR in response to seasonal pollens when studied outside pollen exposure and to compare the cellular profile of their sputum with that of patients with perennial asthma (AS); (2) to assess whether two distinct metachromatic cells, mast cells and basophils, are present in induced sputum; and (3) to explore the relationship between cells in sputum and the degree of bronchial responsiveness.

METHODS

Patients

We selected two groups of consecutive patients who were referred to our outpatient clinic: patients with a history of seasonal AR in response to grass pollen without asthma (AR group) and patients with AS (AS group).

Inclusion criteria for patients with rhinitis were: (1) a history of seasonal rhinitis without cough, wheezing, or shortness of breath during natural exposure, lasting 3 to 5 years; (2) positive skin test responses to grass pollens only; and (3) baseline FEV₁ greater than 80% of predicted value. Presence of bronchial responsiveness to methacholine was not a prerequisite for selection. As a routine procedure, all patients recorded nasal and bronchial symptoms during at least two pollen seasons before this study, and the methacholine PD₂₀ was measured each year outside pollen exposure. None reported asthmatic symptoms during pollen exposure, and methacholine PD₂₀ did not vary significantly. Patients were studied in February, outside the grass pollen season (mid April to mid June).

Inclusion criteria for patients with asthma were clinical

history of asthma as defined by the American Thoracic Society, positive skin test responses to *Dermatophagoides pteronyssinus* only, absence of bronchial symptoms at the time of the study, no asthmatic exacerbation in the preceding 8 weeks, baseline FEV₁ greater than 80% of predicted value, current therapy with only inhaled short-acting β_2 -agonists on an as-needed basis, and presence of bronchial responsiveness to methacholine. Exclusion criteria for both groups were lower or upper respiratory tract infection in the previous 8 weeks, current smoking habit, and history of spontaneous sputum production at the time of recruitment. Patients with asthma treated with inhaled or oral corticosteroids in the 6 months before the study were also excluded.

Presence of atopy was determined by skin prick tests with a standard battery of 12 common airborne allergen extracts (mixed grass pollens, *Compositae* spp., *Parietaria judaica*, *Parietaria officinalis*, birch, *Populus alba*, *D. pteronyssinus*, dog hair, cat fur, *Alternaria tenuis*, *Aspergillus fumigatus*, and *Cladosporium herbarum*).

All patients gave informed consent to participate in the study. The study was performed according to the Helsinki declaration.

Sputum induction and processing

Sputum induction was performed between 8:00 and 10:00 AM, according to the method proposed by Fahy et al.,¹¹ with slight modifications. Before sputum induction, all patients inhaled 2 puffs of salbutamol (200 μ g) and 10 minutes later underwent spirometry. Then patients were asked to inhale nebulized sterile 3% saline solution for 20 minutes from an ultrasonic nebulizer (Mistogen EN143; mean output, 2 ml/min). Patients coughed throughout the procedure and expectorated into a glass container. The sputum sample was homogenized with 5% N-acetyl-L-cysteine added in equal volume. The sample was placed in a shaking bath at 37°C for 30 minutes to ensure complete homogenization. Ten microliters of homogenized sputum was used to determine the total cell count with a Neubauer's hemocytometer. Then sputum was washed three times in saline solution and centrifuged at 300 g for 10 minutes. Suspension was cytocentrifuged at 700 rpm for 5 minutes (Cytospin 3; Shandon Inc., Pittsburgh, Pa.), and slides were air-dried for 6 hours. For immunocytochemical staining, some slides were wrapped in aluminum foil and stored at -80°C until use. Differential cell count was obtained by staining two slides with Diff-Quik (Baxter Scientific Products) and based on 300 cells. Counts of metachromatic cells were made by fixing cells in Carnoy's solution for 1 hour and staining them with 5% alcian blue (pH 2.0), followed by counterstaining with Harris's hematoxylin. It has been demonstrated that it is technically feasible to distinguish two different metachromatic cell types: mast cells are large and pleomorphic with abundant granules and single nuclei, whereas basophils are small and round with few granules and multilobated nuclei.^{16, 23} Differential counts of mast cells and basophils were made by counting 1000 cells. Differential cell counts were always expressed as percentage of total nucleated cells excluding squamous cells.¹¹ The induction of sputum was successfully accomplished in 78% of selected patients with AR and in 82% of selected patients with asthma. Slides were coded and cell counts were performed by an expert observer who did not know the clinical characteristics of patients.

Immunocytochemistry

Tryptase-positive mast cells were stained with a mouse anti-human monoclonal antibody (Chemicon International Inc., Temecula, Calif.). Slides were fixed in Carnoy's solution

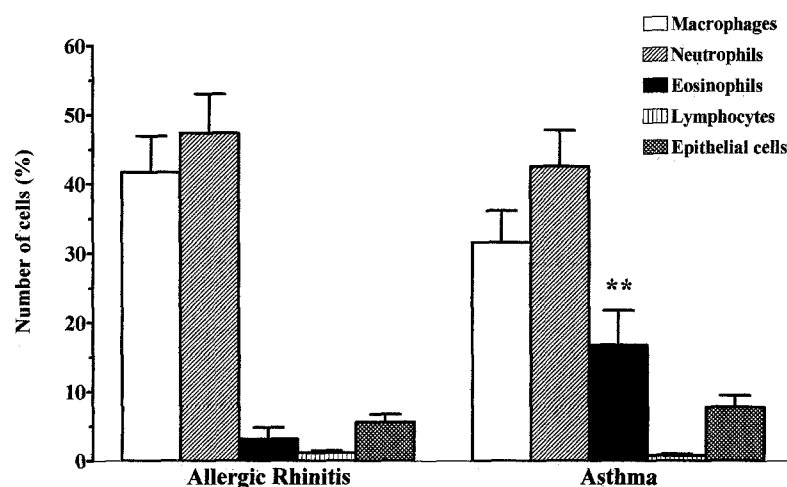


FIG. 1. Differential cell counts in induced sputum from patients with seasonal AR ($n = 30$) and asthma ($n = 15$). Values are means and SEMs. ** $p < 0.001$.

TABLE I. Physiologic and clinical characteristics of subjects

	Allergic rhinitis (AR)			Asthma ($n = 15$)
	Whole group ($n = 30$)	AR- group ($n = 15$)	AR+ group ($n = 15$)	
Gender (M/F)	10/20	6/9	4/11	4/11
Age (yr)	29.3 ± 2.4	30.8 ± 2.8	27.7 ± 1.92	30.9 ± 2.9
FEV ₁ (% pred)	98.8 ± 3.1	97.1 ± 3.4	96.5 ± 2.9	92.6 ± 2.3
Methacholine PD ₂₀ range (μ mol)	2.2 to >24*	>24	2.2 to 19.6*	0.42 to 2.6

Values are expressed as means $\pm$ SEM (age and FEV₁). The AR- group had an unmeasurable methacholine PD₂₀ ($>24 \mu$ mol), whereas the AR+ group had a measurable methacholine PD₂₀.

* $p < 0.001$ compared with asthma.

and incubated with the primary antibody (3 μ g/ml in Tris-buffered saline with 1% bovine serum albumin, pH 7.6) at room temperature. Then the binding was detected by the LSAB, Alkaline Phosphate kit (Dako Corp., Carpinteria, Calif.). The number of positive cells was determined by counting a total of 1000 cells.

Cytospin preparations were also fixed in acetone for 10 minutes, then in chloroform for an additional 10 minutes, and stained with a mouse EG2 anti-human eosinophil cationic protein antibody (Pharmacia, Uppsala, Sweden) diluted at 1 μ g/ml in Tris-buffered saline (pH 7.4) with 1% bovine serum albumin. Antibody binding was displayed by alkaline phosphatase-labeled streptavidin-biotin complexes (Dako LSAB kit, AP). EG2+ cells were estimated by counting 800 cells and expressed as percentage of total cells. Slides incubated with Tris-buffered saline with 1% bovine serum albumin were used as negative controls.

Methacholine challenge

Bronchial response to methacholine was assessed by having subjects inhale increasing concentrations of methacholine delivered by a dosimeter (Mefar MB4, Brescia, Italy) according to a standard procedure. Inhalations were continued until FEV₁ decreased by 20% from postsaline value or until a maximal dose of 24 μ mol was administered. Bronchial response was expressed as methacholine PD₂₀ in micromoles. When methacholine PD₂₀ was not measurable, a PD₂₀ value equal to 24 μ mol was

assigned to the patient. Bronchial challenge was always done 3 days after sputum induction.

Statistical analysis

Number of cells was reported as mean $\pm$ SEM. Methacholine PD₂₀ values were logarithmically transformed before the analysis. Results were tested for normality by the Shapiro-Wilk test. Differences in gender between groups were examined by Fisher's exact test. Differences among groups were examined by Kruskal-Wallis analysis of variance by ranks or by analysis of variance. If differences were significant, post hoc analysis was performed with the Mann-Whitney U test or the Student-Newman-Keuls test. Between-observer and within-sample reproducibility of cell counts was assessed by the intraclass correlation coefficient. Cell counts in induced sputum were not normally distributed. Therefore correlations were assessed by Spearman's rank method. A p value of less than 0.05 was considered significant. All statistical tests were performed with Unistat 3.0 statistical software (Megalon S.A., Novato, Calif.).

RESULTS

Characteristics of patients with AR and asthma, who successfully accomplished sputum induction, are shown in Table I. We studied 30 patients with AR and 15 patients with asthma. Four patients in each group were former smokers. Seven patients with asthma were using

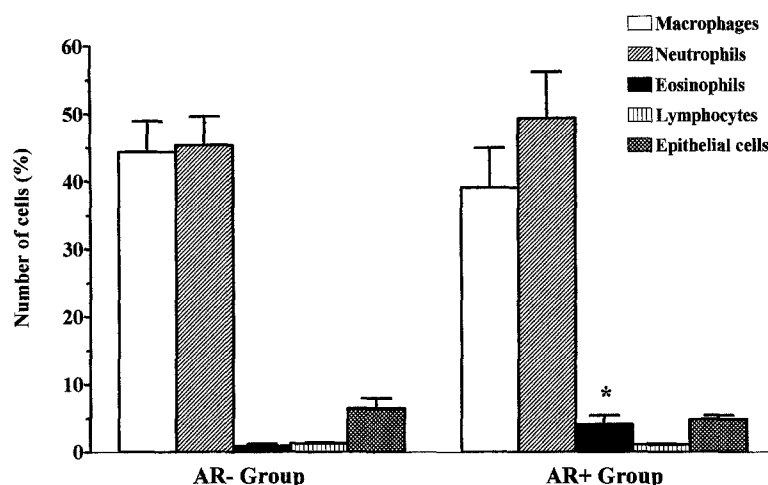


FIG. 2. Differential cell counts in induced sputum from patients with seasonal AR without (AR- group, $n = 15$) and with (AR+ group, $n = 15$) measurable bronchial responsiveness to methacholine. Values are means and SEMs. * $p < 0.05$.

TABLE II. Counts of eosinophils and metachromatic cells and immunocytochemistry in induced sputum from patients with AR and AS

	Eosinophils (%)	EG2+ cells (%)	Mast cells (%)	Basophils (%)	Tryptase+ cells (%)
AR ($n = 30$)	$2.49 \pm 1.0^*$	$0.41 \pm 0.21^\dagger$	$0.03 \pm 0.02^\ddagger$	$0.06 \pm 0.04^\S$	ND
AR- group ($n = 15$)	$0.87 \pm 0.34^\S $	$0^\ddagger $	0.03 ± 0.03	$0.04 \pm 0.02^\ddagger$	ND
AR+ group ($n = 15$)	$4.11 \pm 1.32^\ddagger$	0.83 ± 0.26	0.04 ± 0.02	0.08 ± 0.05	ND
Asthma ($n = 15$)	16.7 ± 5.06	2.90 ± 0.81	0.13 ± 0.05	0.36 ± 0.14	0.09 ± 0.02

Values are expressed as means $\pm$ SEM.

ND, Not done.

* $p < 0.001$ compared with asthma.

$^\dagger p < 0.02$ compared with asthma.

$^\ddagger p < 0.05$ compared with asthma.

$^\S p < 0.01$ compared with asthma.

$|| p < 0.05$ compared with AR+ group.

inhaled β_2 -agonists as needed. The two groups were not different in regard to gender, age, and baseline FEV₁. Methacholine PD₂₀ values in the AR group were different from those of the AS group ($p < 0.001$, Table I). Patients in the AR group were divided according to absence or presence of measurable methacholine PD₂₀. Methacholine PD₂₀ was not measurable in 15 patients with AR (AR- group) and ranged between 2.2 and 19.6 μ mol in the remaining 15 patients (AR+ group). Characteristics of the two subgroups of patients with AR are also reported in Table I.

Differential cell counts in induced sputum and immunocytochemistry

There was no difference between groups in the number of total cells in sputum (AR group, 189.9×10^4 ; AS group, 218.7×10^4). Moreover, the percentage of macrophages, neutrophils, lymphocytes, and epithelial cells in each group was not statistically different (Fig. 1). The mean percentage of eosinophils in the AS group was approximately sixfold greater than that in the AR group

($p < 0.001$, Fig. 1 and Table II). When differences between the two subgroups of patients with AR were examined, the percentage of eosinophils was significantly higher in the AR+ group compared with the AR- group ($p < 0.05$, Fig. 2 and Table II).

Cytospin slides stained with alcian blue confirmed the presence of two morphologically different types of metachromatic cells. The two types of metachromatic cells were clearly recognizable: mast cells had a round shape and single nuclei, and basophils were smaller with an oval shape and multilobated nuclei. When unclassified cells were present, their percentage was always lower than 10% of metachromatic cells. Intraclass correlation coefficients for between-observer ($n = 10$) and within-sample ($n = 15$) assessments of mast cell and basophil counts were 0.88 and 0.91 and 0.92 and 0.95, respectively.

Patients with asthma had significantly more mast cells only when compared with all patients with AR ($p < 0.05$, Table II), whereas differences between the two AR subgroups were not significant. Moreover, patients with

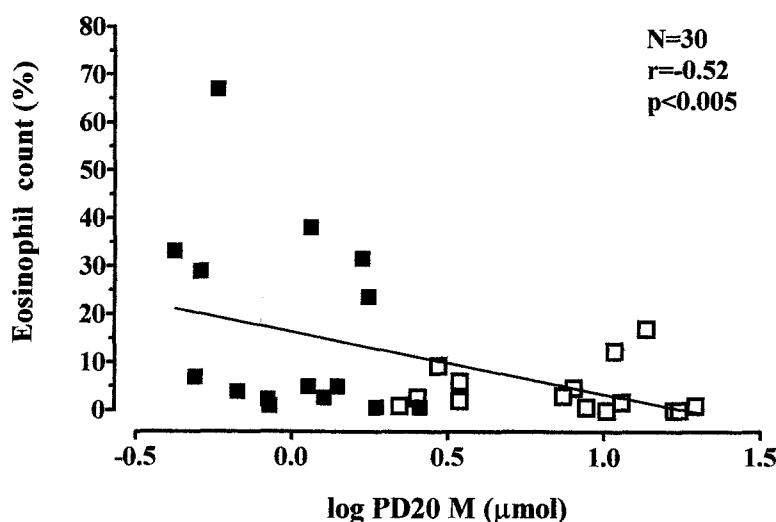


FIG. 3. Correlation between methacholine PD₂₀ and eosinophils in induced sputum from patients in AR+ group (open squares, $n = 15$) and AS group (filled squares, $n = 15$). r , Spearman's rank correlation coefficient.

AS had significantly more basophils than all patients with AR ($p < 0.01$, Table II) and patients in the AR-group ($p < 0.05$, Table II). The difference between the two AR subgroups was not significant (Table II). Basophils could be counted in only seven of 30 patients with AR and in eight of 15 patients with asthma. The mean number of basophils in induced sputum from patients with AR and asthma was approximately two to three times higher than the mean number of mast cells.

Immunocytochemistry demonstrates that tryptase-positive cells have the same structures as mast cells and that the percentage of tryptase-positive cells was nearly equal to that of mast cells in alcian blue-stained slides (Table II). Immunocytochemistry revealed that a low proportion of eosinophils in induced sputum stained positively with EG2 monoclonal antibody. Patients with asthma had significantly more EG2+ cells when compared with all patients with AR ($p < 0.02$, Table II) and with patients in the AR-group ($p < 0.05$, Table II), but not when compared with the AR+ group. In the AR+ group, the mean count of EG2+ cells was higher than that in the AR- group ($p < 0.05$, Table II). EG2 cells could be counted in sputum obtained from seven of 30 patients with rhinitis and in nine of 15 patients with asthma.

Cell counts and bronchial responsiveness to methacholine

The relationship between differential counts of inflammatory cells in sputum and methacholine PD₂₀ was examined in the AR+ and AS groups. In the whole population ($n = 30$), there was an inverse correlation between methacholine PD₂₀ and eosinophils in induced sputum ($r = -0.52$, $p < 0.005$; Fig. 3). Moreover, there was a weak but significant correlation between methacholine PD₂₀ and mast cells ($r = -0.44$, $p < 0.02$; Fig. 4) but not with basophils ($r = -0.29$, $p > 0.05$).

DISCUSSION

In this study we demonstrated that eosinophils and metachromatic cells are present in induced sputum from patients with seasonal AR even outside exposure to the sensitizing antigen. In these patients, cell counts differed from those of patients with asthma. We found that metachromatic cells in induced sputum had the features of both mast cells and basophils. An important and new observation of this study was that basophils were present in seven of 30 (23%) sputum samples from patients in the AR group and in eight of 15 (53%) sputum samples from patients with asthma. Moreover, we showed that the percentage of eosinophils, EG2+ cells, and basophils significantly increases or tends to increase with bronchial responsiveness to methacholine. Finally, we found that methacholine PD₂₀ values significantly correlate with eosinophil and mast cell counts.

Bronchial asthma is characterized by the recruitment of eosinophils and mast cells within the airways,^{1,2} whereas the presence of airway inflammation in AR is still debated. Previous studies of BAL cells failed to demonstrate any difference between normal subjects and patients with AR studied outside the pollen season.^{6,17} However, other studies showed that the number of eosinophils in BAL and bronchial biopsy specimens from patients with AR is intermediate and different from that in BAL and biopsy specimens from normal subjects and patients with asthma.^{8,9} Our results confirm that both metachromatic cells and eosinophils are present in induced sputum from patients with asthma.^{10,11} Moreover, they demonstrate that these cells are also present in sputum from patients with seasonal AR, even outside pollen exposure. This observation is of particular interest because presence and degree of a "chronic" inflammation, at least in some patients, may indicate an important predisposition to the development of bronchial symptoms during natural pollen exposure.

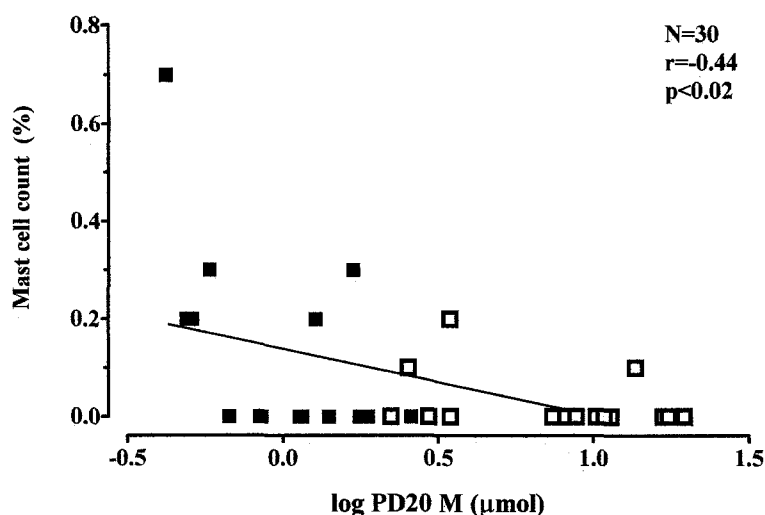


FIG. 4. Correlation between methacholine PD₂₀ and mast cells in induced sputum from patients in AR+ group (open squares, n = 15) and AS group (filled squares, n = 15). r, Spearman's rank correlation coefficient.

Mast cells are highly specialized effector cells of the immune system, and together with eosinophils, constitute the hallmark of allergic inflammation.^{1,2} Cytoplasmic granules of both cell types show metachromasia after appropriate fixation (in Carnoy's solution) and staining (alcian blue). On the basis of differences in cell morphologic features, we demonstrated that metachromatic cells in induced sputum included both mast cells and basophils. We found that only large metachromatic cells with single nuclei (i.e., mast cells) exhibited immunoreactivity for tryptase. We therefore reasoned that metachromatic cells with smaller size and lobated nuclei are basophils. The estimate of mast cell number in induced sputum from patients with asthma is in agreement with previous reports.¹² Moreover, our study clearly shows that basophils are seen in sputum from most patients with asthma and from a few patients with AR. The percentage of basophils in patients with asthma was about threefold greater than the percentage of mast cells. The concept that basophils are recruited into bronchial airways of patients with asthma is still debated. Basophils have been found in spontaneously produced sputum from patients with asthma and have been found to be increased with the severity of symptoms.²⁴ Gibson et al.¹⁰ recognized basophils in induced sputum from only one of 10 patients with asthma. However, they studied patients treated with inhaled corticosteroids or sodium cromoglycate, and the cell count was based on the examination of too few microscopic fields. These factors may have significantly limited cell counts because the basophil count is markedly reduced by both drugs,²⁵ and more cells need to be counted for an accurate estimate. Several pieces of evidence suggest that basophils play an important role in sustaining airway inflammation.²⁶ Circulating basophils²⁷ and basophils in BAL¹⁶ increase during asthma exacerbation. Moreover, basophils comprise more than 95% of all metachromatic cells in BAL during the late asthmatic response^{17,18} and

are present in lung tissue of patients who died of asthma.¹⁵ Therefore the presence of basophils in induced sputum samples from some of our subjects is in keeping with the hypothesis that basophils may contribute to airway inflammation.

Our study clearly demonstrates that bronchial responsiveness is associated with the presence of metachromatic cells and eosinophils in induced sputum. Our finding that the number of metachromatic cells, eosinophils, and EG2+ cells in the AR+ group is intermediate compared with that found in the AR- and AS groups supports the view that the inflammatory process is important in the induction of bronchial responsiveness. Previous reports have failed to demonstrate any difference in the number of metachromatic cells,^{6,17} eosinophils,^{6,17} and EG2+ cells⁸ in BAL of patients with AR and normal control subjects. However, too few patients were included in the study populations.

Our finding of a weak but significant correlation between mast cells and eosinophils and methacholine PD₂₀ supports the concept that bronchial responsiveness is a rather complex mechanism. It is certainly sustained not only by the recruitment of inflammatory cells but also by the release of their mediators, by the interaction with the epithelium,²² and by the remodeling of the airway walls.²⁸ A significant correlation between bronchial responsiveness and number of eosinophils and mast cells in sputum^{12,29} and BAL^{19,30} and number of intraepithelial eosinophils in bronchial biopsy specimens²⁸ has been previously ascertained. We were unable to find any significant correlation between EG2+ cells and bronchial responsiveness. Our results are therefore at variance with those reported by Bradley et al.⁸ They showed a weak but significant correlation between EG2+ cells in bronchial biopsy specimens from patients with asthma showing a methacholine PC₂₀ lower than 8 mg/ml. Differences in patients' characteristics and technical factors may explain this discrepancy.³¹

In conclusion, we have demonstrated that inflammatory cells are present in induced sputum of patients with seasonal AR, even outside natural exposure to pollens. Because the number of metachromatic cells and eosinophils in the AR- group is different from that of patients with asthma and is intermediate in the AR+ group, our results not only confirm the hypothesis that inflammation is important in sustaining the presence of bronchial responsiveness in perennial asthma, but also indicate that they may be involved in its pathogenesis in seasonal AR.

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