

Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease

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Background: Eosinophils but not neutrophils may play a role in the airway inflammation of asthma. In chronic bronchitis (CB) and chronic obstructive pulmonary disease (COPD), neutrophils are present in the airways. To differentiate among the pathology of asthma, CB, and COPD eosinophils and neutrophils were studied in peripheral blood, bronchial biopsy specimens, and bronchoalveolar lavage fluid (BALF).

Methods: We studied nine nonsmoking healthy subjects, 20 nonsmoking patients with asthma, 10 nonatopic smoking patients with CB (forced expiratory volume in 1 second: $98.4\% \pm 11.3\%$) and 17 patients with COPD (forced expiratory volume in 1 second: $51.2\% \pm 14.3\%$). Eosinophils were characterized by their enumeration in biopsy specimens (EG2 monoclonal antibody), peripheral blood, and BALF and by measurement of eosinophil cationic protein in BALF. Neutrophils were characterized by their enumeration in biopsy specimens (anti-elastase monoclonal antibody) and BALF and by measurement of neutrophil-specific myeloperoxidase in BALF.

Results: In patients with asthma we found degranulated eosinophils in biopsy specimens and significantly increased eosinophil cationic protein levels in BALF. In patients with CB or COPD, eosinophil numbers in biopsy specimens were not significantly different from those of patients with asthma, but cells were not degranulated and eosinophil cationic protein levels in BALF were similar to those of normal subjects. In patients with CB or COPD neutrophils were not increased in the mucosa, but neutrophil numbers and myeloperoxidase levels in BALF were significantly increased.

Conclusion: The percentages of neutrophils in BALF were greater in patients with COPD than in those with CB, suggesting a role in the chronic airflow limitation. (*J ALLERGY CLIN IMMUNOL* 1993;92:537-48.)

Asthma and chronic bronchitis (CB) have been recognized for a long time; however, their definitions are still a matter of debate, although many attempts to characterize them have been made.¹ To distinguish chronic obstructive pulmonary disease (COPD) from asthma, the American Thoracic Society suggested that "COPD be defined as a disorder characterized by abnormal tests of expiratory flow that do not change over periods of

Abbreviations used

BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
CB:	Chronic bronchitis
COPD:	Chronic obstructive pulmonary disease
ECP:	Eosinophil cationic protein
FEV ₁ :	Forced expiratory volume in 1 second
HE:	Hematoxylin-eosin
MPO:	Myeloperoxidase

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Received for publication Aug. 25, 1992; revised Mar. 23, 1993; accepted for publication Mar. 23, 1993.

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several months observation."¹ In the same statement it was indicated that patients with fatal asthma had evidence of eosinophilic inflammation in the airways. However, it may be difficult to differentiate some patients with long-standing

asthma who show poor reversibility of airflow obstruction from others with CB or COPD who have significant reversibility of airflow obstruction and bronchial hyperreactivity.² The availability of results of bronchial biopsies carried out during fiberoptic bronchoscopy made it possible to better characterize asthma, and major features observed included epithelial shedding and eosinophilic inflammation of the airways^{3, 4}; therefore, several recently published consensus on asthma added these pathologic features to the definition of asthma.

Symptoms of CB are at least in part due to bronchial inflammation. Its pathologic characteristics appear to differ largely from those of asthma because in CB there is epithelial metaplasia, and inflammation may be more related to neutrophils and macrophages.^{5, 6} Eosinophils have been inconsistently found in the airways of patients with CB,^{7, 8} and there was no correlation between the presence of these cells and either airway reversibility after inhalation of β -agonists or results of the methacholine challenge.⁹ These studies were difficult to analyze fully because they were done with the use of conventional histologic tests and did not use immunohistochemistry, which has been found to improve the characterization of cells in the airways. Bronchoalveolar lavage (BAL) studies carried out in patients with CB showed an increased number of total cells and neutrophils.^{10, 11}

Airway inflammation can be studied by combining results of bronchial biopsies with those of BAL.⁴ Biopsies directly evaluate inflammatory cells and airway damage, but because they are carried out on large airways, they do not provide reliable quantitative data and do not recognize the possible heterogeneity of airway lesions. BAL indirectly appreciates airway inflammation, provides information on a wider part of the airways, and is quantitative. However, BAL too has its drawbacks. The technique is random, there may be mixing of bronchial and alveolar cells, there may be mediator degradation in the fluid, and some mediators may be shared by different inflammatory cell populations. Bronchoalveolar lavage fluid (BALF) fractionation may provide more accurate data on the anatomic site of the lung being examined; the first lavage sample is more related to bronchi and further samples are more related to bronchioles and alveoli.¹²

Some of the cells that participate in airway inflammation and release of specific mediators include eosinophils and neutrophils. Eosinophils

contain in their granules eosinophil cationic protein (ECP),⁴ and neutrophils contain myeloperoxidase (MPO) stored in the azurophil granule and released during phagocytosis, or cell activation elastase.¹³ Because both cell types release these mediators during activation, it is possible to study this activation by titrating these mediators in BALF and characterizing them in bronchial biopsy specimens with immunohistochemical techniques.¹⁴

In an attempt to compare the inflammation of CB with that of asthma, we studied eosinophils and neutrophils in 20 patients with asthma, 27 patients with CB, and 9 normal individuals. All subjects had the same investigations, which combined peripheral blood, bronchial biopsy, and BALF studies. Eosinophils and neutrophils were enumerated in peripheral blood and BALF. The structure of the bronchi (epithelium, basement membrane) was studied after hematoxylin-eosin (HE) staining. Eosinophils were characterized in the bronchi by EG2 monoclonal antibodies and by the measurement of eosinophil cationic protein (ECP) in the BALF. Neutrophils were characterized in the bronchi by elastase monoclonal antibodies and by the measurement of myeloperoxidase in the BALF.

METHODS

Subjects

We studied 20 patients with asthma (20 to 59 years old; mean $\pm$ SD: 40.3 $\pm$ 13.9 years). Asthma was defined according to the criteria of the American Thoracic Society¹: all patients had either a reversible airway obstruction characterized by an increase of at least 15% in the forced expiratory volume in 1 second (FEV₁) after inhalation of 200 μ g of salbutamol or a positive inhalation challenge with carbachol. The carbachol inhalation challenge was carried out at least 2 weeks before bronchoscopy in patients who had a baseline FEV₁ over 80% of predicted values. The severity of asthma was assessed by the Aas score as previously explained.¹⁵ None of the subjects were current or previous smokers. Patients were excluded from the study if they had had a severe exacerbation of asthma that necessitated a hospital stay during the month preceding the study.

Twenty-seven patients with CB (22 to 72 years old; mean $\pm$ SD: 54.8 $\pm$ 12.9 years) were tested. CB was defined according to the criteria of the American Thoracic Society¹: cough and sputum on most days of the month for at least 3 months a year during the previous 2 years. Because an overlap with asthma may exist in subjects with so-called "asthmatic bronchitis,"¹² patients who showed one of the following features were excluded: a history of perennial allergic rhinitis and/or

a positive diagnosis of immediate-type hypersensitivity (as described below), wheezing, an improvement in FEV₁ of over 10% after inhalation of 200 µg of salbutamol or a positive inhalation challenge with carbachol (carried out in patients with FEV₁ over 70% of predicted values). Patients with obvious emphysema, as assessed by routine chest x-ray studies were also excluded. Smoking was carefully checked, and a minimum of 15 pack-years smoked was required (15 to 100 pack-years, mean $\pm$ SD: 41.2 $\pm$ 22.5 pack-years). COPD was defined as a disorder characterized by abnormal test results for expiratory flow, and although there is no definitive cutoff limit, patients with FEV₁ under 70% of predicted values were classified as having COPD.¹ Patients with FEV₁ over 80% of predicted values were classified as having CB because there was no patient with FEV₁ that ranged between 70% and 80%.

Nine normal healthy volunteers (22 to 57 years old, mean $\pm$ SD: 37.2 $\pm$ 11.1 years) were used as a control group. Their pulmonary function was within the normal range. They were nonallergic and had never had asthma. None of the subjects were current or former smokers.

No subject had any bronchial or respiratory tract infection during the month preceding the test. Patients were excluded from the study if they had taken systemic corticosteroids of any form during the previous 2 months; inhaled corticosteroids the month before; nedocromil sodium, sodium cromoglycate or ketotifen in the previous 48 hours. β_2 agonists were only withdrawn for 8 hours before the study because they are unlikely to affect eosinophils.

The research was carried out with the consent of the patients and approval of the Ethics Committee of our institution.

Investigations

Pulmonary function test. A pulmonary function test was carried out the day before the bronchoscopy with the use of a flow-volume loop done with a Pneumoscreeen (E. Jaeger Laboratories, Würzburg, Germany).

Enumeration of blood eosinophils. Blood eosinophils were counted with a Technicon Hemalog (Technicon, Tarryton, N.Y.) on the same day as the pulmonary function test.

Diagnosis of immediate-type hypersensitivity. All subjects underwent identical investigations. Allergy tests, including a battery of extracts based on the common food and aeroallergens found in the Montpellier area, were performed with the skin prick method. Total serum IgE (Phadebas PRIST and Phadiatop [Pharmacia Diagnostics AB, Uppsala, Sweden]) completed the study. Serum-specific IgE was titrated by the Phadebas RAST (Pharmacia Diagnostics AB) in patients with positive skin prick test results.

Fiberoptic bronchoscopy. Fiberoptic bronchoscopy was carried out as previously described.⁴ Briefly, after premedication with atropine and diazepam and local

anesthesia with 2% lidocaine applied to the upper respiratory tract, a BFTR Olympus fiberoptic bronchoscope (Olympus, Paris, France) was inserted into the trachea. BAL was carried out in one of the subsegmental bronchi of the middle lobe by first injecting a 50 ml aliquot and then four aliquots of sterile saline solution, which were reaspirated by gentle syringe suction. Because more than 50% of patients with CB had trapping of the first aliquot, analyses were only carried out on the subsequent aliquots. Each bronchoscopy was carried out with oxygen and epinephrine readily available; all patients had intravenous access.

The procedure was carried out in patients who were in various stages of COPD and in some patients with severe asthma, although the current recommendations favor the use of bronchoscopies in patients with mild asthma.¹⁶ The safety aspect of this research was therefore discussed in detail before the study began, and it was approved by the ethics committee of the hospital. Moreover, in patients with asthma we have shown that there was no correlation between the level of FEV₁ and the safety of bronchoscopy.¹⁷

Immediately after lavage, mucus was removed from the fluid by filtration through gauze. Cell counts were made on BALF before further processing. Then BALF was centrifuged at 400 g for 10 minutes at +4° C, and the supernatant was frozen at -80° C until required later.

Examination of BAL cells. The BAL cytologic study was done on cytocentrifuged slides (Cytospin; Shandon Scientific Ltd., Runcorn, Cheshire, England) and stained by May-Grünwald Giemsa stain.

Titration of mediators. Eosinophil cationic protein (ECP) was measured in BALF by a double antibody radioimmunoassay (Pharmacia Diagnostics AB) with a polyclonal rabbit antibody as previously described. ECP in samples competes with a fixed amount of iodine-125-labeled ECP for the binding sites of specific antibodies. The ECP standards are calibrated against pure ECP prepared according to the method of Venge et al.¹⁸ The technique used was carried out according to the package insert. Levels less than 2 µg/L are undetectable.

Myeloperoxidase was measured with a double antibody radioimmunoassay (Pharmacia Diagnostic AB).¹⁹ MPO in samples competes with a fixed amount of ¹²⁵I-labeled MPO for the binding sites of a specific polyclonal rabbit antibody. The technique used was as stated on the package insert. The interassay coefficient of variation was under 12%, and levels under 8 µg/L are undetectable.

Bronchial biopsies. Biopsy specimens were fixed in 10% formaldehyde (pH 7.2) and embedded in paraffin blocks. Six-micrometer tissue sections were affixed to microscope slides with Le Page's glue, and after deparaffination and rehydration, the slides were ready for immunohistochemistry. HE staining was then carried out as previously described.⁴

TABLE I. Characteristics of patients

	Control subjects	Patients with CB	Patients with COPD	Patients with asthma
Number	9	10	17	20
Age (yr)	37.2 $\pm$ 11.1	43.3 $\pm$ 12.8	62.9 $\pm$ 7.4	40.3 $\pm$ 13.9
FEV ₁	98.6 $\pm$ 11.7	98.4 $\pm$ 11.3	51.2 $\pm$ 14.3	78.3 $\pm$ 21.1
Blood eosinophils/mm ²	105.9 $\pm$ 58.4	277.3 $\pm$ 205.2	256.8 $\pm$ 111.3	500.1 $\pm$ 284.1
BAL fluid analyses				
Recovery (%)	56.5 $\pm$ 11.3	44.3 $\pm$ 12.0	20.7 $\pm$ 7.2	37.7 $\pm$ 10.7
Cell number (/μl)	143.0 $\pm$ 55.9	320.4 $\pm$ 172.5	296.8 $\pm$ 417.0	133.8 $\pm$ 65.1
Macrophages (%)	91.1 $\pm$ 6.2	88.1 $\pm$ 6.8	55.2 $\pm$ 35.5	88.5 $\pm$ 11.2
Lymphocytes (%)	8.3 $\pm$ 6.1	4.8 $\pm$ 4.1	3.5 $\pm$ 4.2	7.7 $\pm$ 9.4
Eosinophils (%)	0.07 $\pm$ 0.27	1.1 $\pm$ 2.7	1.0 $\pm$ 1.7	1.6 $\pm$ 2.5
Neutrophils (%)	0.5 $\pm$ 0.65	4.9 $\pm$ 4.7	33.8 $\pm$ 39.3	1.5 $\pm$ 2.6
Epithelial cells (%)	0.25 $\pm$ 0.9	0.3 $\pm$ 0.7	5.2 $\pm$ 8.3	0.5 $\pm$ 2.1
ECP (μg/L)*	0	0-2.2	0-11.3	0-71
MPO (μg/L)*	0-10	0-28	0-174	0-30
Bronchial biopsies				
Eosinophils (/mm ²)†	0.09 (0-0.08)	0.3-4.9	0.2-5.5	14.5 (0-50)
Degranulation score*	0	0-2	0-2	0-3
Neutrophils (/mm ²)†	0.0 (0-3.4)	0-0.5	0-7.6	0.6 (0-16)
Degranulation score*	0	0-1	0-2	0-2

Results are expressed as means $\pm$ SD, median, * or median and range†.

On specimens prepared similarly, two monoclonal antibodies were used: antibody EG2 (Pharmacia Diagnostics AB) specific for a common epitope on ECP and eosinophil-derived neurotoxin and recognizing secreted proteins and antibody specific for neutrophil elastase (Dako Corp., Carpinteria, Calif.).¹³ We did not use an antibody against MPO because it cannot be used in paraffin-embedded sections. Immunohistochemistry on bronchial tissue sections was carried out with an alkaline phosphatase-linked rabbit anti-mouse antibody as a second antibody. Control slides were treated with unrelated IgG₂ mouse antibody of the same IgG isotype. Bronchial specimens from normal subjects, patients with CB and patients with asthma were run in the same experiment series because small differences may be seen when experiments are run at different times.

Biopsy specimens were not studied when their size was too small or when submucosa was absent, the sample being shed epithelium. Thus only seven normal subjects, 24 patients with CB, and 13 patients with asthma had an immunohistochemical study.

The evaluation was made by two independent pathologists who were unaware of the patients' data. All biopsy samples were coded, and sections were studied in a blinded fashion. Cellular infiltration of eosinophils and neutrophils, as assessed by cellular staining, was scored by number of cells per square millimeter. The number of positively stained cells was counted in a deep zone, defined by a squared eyepiece, along the length of the epithelial membrane. Because of the desquamation of epithelium, cells present in epithelium were not considered. Next, we scored the degree of extracellular deposition of ECP and elastase on a scale

ranging from 0 to 3 according to the method of Fujisawa et al.¹⁴ Specimens with no or occasional extracellular staining were scored as 0. If there were scattered foci containing faint staining, the specimen was rated as 1. Specimens with scattered foci containing marked staining were rated as 2, and 3 was assigned to specimens showing intense deposition throughout the lesion.

Statistical analysis

Statistical analyses were carried out by means of nonparametric tests. The Kruskal-Wallis test was used to compare the four groups of subjects (patients with asthma, CB, and COPD and control subjects). Then the Mann-Whitney U test with Bonferroni's correction was used to compare the different groups. Results are given as means $\pm$ SD or as medians with range.

RESULTS

Characteristics of the patients

The demographic characteristics of the subjects are presented in Table I and the statistical analyses in Table II. The mean age of patients with asthma, patients with CB, and normal subjects was similar, but it was significantly higher in patients with COPD. The pulmonary function of normal subjects was within normal range. FEV₁ of patients with CB was similar to that of normal subjects. Patients with asthma had a significant decrease in FEV₁ (38% to 110% of predicted values, mean $\pm$ SD: 78.3% $\pm$ 21% of predicted

TABLE II. Statistical analysis

	Kruskal-Wallis	C/BC	C/COPD	C/A	BC/COPD	BC/A	COPD/A
Age	$p < 0.03$	NS	$p < 0.001$	NS	$p < 0.001$	NS	$p < 0.001$
FEV ₁	$p < 0.01$	NS	$p < 0.001$	$p < 0.005$	$p < 0.001$	$p < 0.008$	$p < 0.006$
Blood eosinophils	$p < 0.01$						
BAL fluid analyses							
Recovery	$p < 0.01$	NS	$p < 0.001$	$p < 0.002$	$p < 0.001$	NS	$p < 0.001$
Cell number	$p < 0.04$	$p < 0.007$	NS	NS	NS	$p < 0.006$	NS
Macrophages	$p < 0.01$	NS	$p < 0.001$	NS	$p < 0.007$	NS	$p < 0.001$
Lymphocytes	NS						
Eosinophils	NS						
Neutrophils	$p < 0.01$	$p < 0.008$	$p < 0.002$	NS	NS	NS	$p < 0.008$
ECP	$p < 0.01$	NS	NS	$p < 0.001$	NS	$p < 0.003$	$p < 0.007$
MPO	$p < 0.05$	$p < 0.008$	$p < 0.006$	NS	NS	NS	NS
Bronchial biopsies							
Eosinophils	$p < 0.01$	$p < 0.005$	$p < 0.005$	$p < 0.001$	NS	NS	NS
Neutrophils	NS						

The overall statistical analysis was performed with the Kruskal-Wallis test, and the intergroup analysis was performed with the Mann Whitney U test with Bonferroni's correction.

C, Control subjects; A, asthma; NS, not significant.

values) in comparison with normal subjects and patients with CB. Patients with COPD had a significant decrease of FEV₁ (35% to 69% of predicted values; mean $\pm$ SD: 51.2% $\pm$ 14.3% of predicted values) by comparison with the other three groups. Patients with asthma had variable severity of the disease (Aas score 1: five patients; Aas score 2: six patients; Aas score 3: five patients and Aas 4: four patients) and 60% of patients with asthma were sensitive to perennial allergens.

Peripheral blood eosinophils

The total number of peripheral blood eosinophils (Fig. 1 and Table I) was significantly increased in patients with asthma and patients with CB or COPD in comparison with normal subjects ($p < 0.0001$ and $p < 0.001$, respectively [Mann-Whitney U test]). Moreover, there was a significant increase in patients with asthma in comparison with patients with CB or COPD ($p < 0.001$ [Mann-Whitney U test]). There was a significant correlation in patients with asthma between FEV₁ and eosinophil counts (Rho = 0.61, $p < 0.003$ [Spearman's rank correlation coefficient]). Mean eosinophil counts were similar in patients with CB and COPD.

BAL cells

There was complete BALF trapping in one patient with CB and three patients with COPD. Three others displayed an absence of cells on cytospin, and most of them had severe airway

obstruction. The recovery of BALF (Table I) was significantly greater in the normal subjects than in patients with asthma, CB, or COPD.

The number of cells and the differential counts in normal subjects and in patients with asthma, CB, and COPD are shown in Tables I and II and in Fig. 2. Eosinophils were increased in patients with asthma, CB, and COPD in comparison with normal subjects. There was a significant correlation between eosinophil counts in BALF and FEV₁ in patients with asthma (Rho = 0.56, $p < 0.001$ [Spearman's rank correlation coefficient]) but not in patients with CB or COPD. Percentages of epithelial cells were elevated in the COPD group compared with the three other groups. The percentage of neutrophils was significantly increased in subjects with CB or COPD in comparison with normal subjects. Patients with COPD had an increased number of neutrophils in comparison with patients with CB, but the difference was not significant when Bonferroni's correction was applied. In patients with CB and COPD, there was no correlation between smoking and any of these counts or between eosinophil counts in peripheral blood and BALF or between neutrophil counts in peripheral blood and BALF.

Mediator levels in BALF

Results of mediator level determinations are presented in Fig. 3 and in Table I and II. ECP levels were detectable in none of the normal subjects, in 4 of 20 patients with CB or COPD,

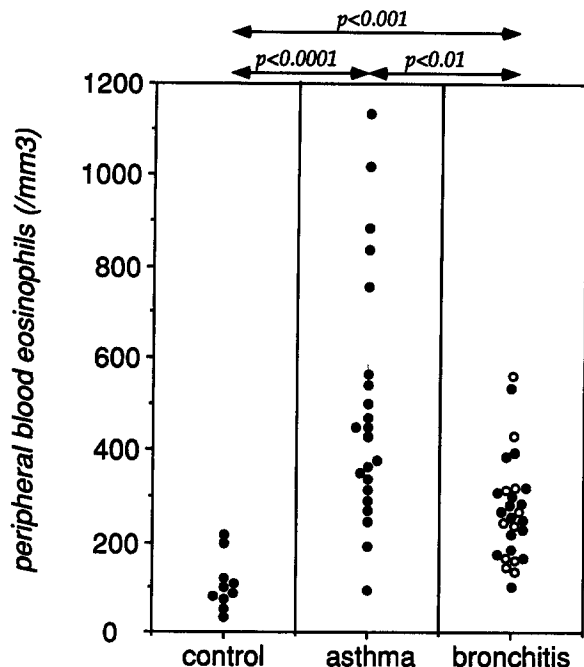


FIG. 1. Peripheral blood eosinophils in patients with bronchitis; open circles represent CB and filled circles represent COPD.

and in 17 of 20 patients with asthma. There was a significant increase in mean ECP levels in patients with asthma in comparison with normal subjects and patients with CB or COPD. There was a significant correlation between ECP in BALF and FEV₁ in patients with asthma ($Rho = 0.60$, $p < 0.005$; Spearman's rank correlation coefficient).

MPO levels were detectable in 1 of 9 normal subjects, 3 of 20 patients with asthma, and 14 of 20 patients with CB or COPD. There was a significant increase in MPO levels in patients with CB or COPD in comparison with normal subjects. The difference in MPO levels did not reach significance when patients with CB or COPD were compared with patients with asthma because of Bonferroni's correction. MPO levels were correlated with percentage of neutrophils in BAL of patients with CB or COPD ($Rho = 0.6$, $p < 0.02$; Spearman's rank correlation coefficient). In patients with CB or COPD there was no correlation between smoking (number of pack-years) and MPO levels.

Immunohistochemistry of bronchial biopsies

HE staining showed striking differences in the epithelium among the three groups of subjects. In normal subjects epithelium was normal in one

subject, partly shed in four, and completely shed in the two others; basement membrane size was normal in all subjects. In patients with asthma, epithelium was completely shed in eight patients, partly shed in four, and not shed in one. One patient had a basal cell hyperplasia. The basement membrane was pseudothickened in all subjects. In patients with CB epithelium was completely shed in three patients, partly shed in two, and not shed in four. In patients with COPD, epithelium was completely shed in four patients, partly shed in five, and not shed in five. Basement membrane size was increased in most patients, but to a lesser extent than in asthma.

Results of immunohistochemistry with EG2 are presented in Figs. 4 and 5. In normal subjects, very few cells reacted with EG2; these cells were found deep in the submucosa and were never degranulated. In four patients with asthma (degranulation score = 3) the cell counts were difficult because of the intense degranulation. The number of eosinophils staining by EG2 was significantly greater in the biopsy specimens from patients with asthma, CB, and COPD in comparison with normal subjects, but the difference among patients with asthma, CB, and COPD was not significant. Eosinophils of patients with asthma were often degranulated with extracellular release of ECP beneath the basement membrane (Figs. 4, A and 6). There was no difference in the eosinophil degranulation between patients with atopic asthma and those with nonatopic asthma. Eosinophils from patients with CB and COPD were less degranulated than those from patients with asthma (Figs. 4, B and 6). Another difference between patients with asthma and those with CB or COPD was the localization of eosinophils. In patients with asthma cells were beneath the basement membrane, whereas in patients with CB or COPD they were localized more deeply in the submucosa. In patients with CB or COPD, when the epithelium was present, eosinophils were never degranulated. In patients with asthma, there was no significant correlation between the severity of asthma assessed by the Aas score or FEV₁ and the number of eosinophils in the biopsy specimens nor between the numbers of eosinophils in the biopsy specimens and eosinophils or ECP levels in the BALF. In patients with CB there was no correlation between smoking (pack-years smoked) and neutrophils or eosinophils in the biopsy specimens.

There was no significant difference among the

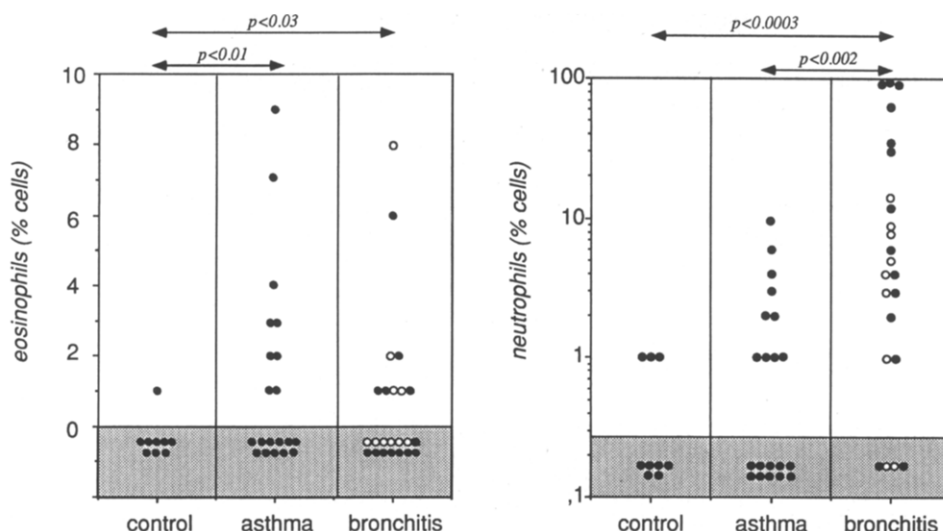


FIG. 2. BAL eosinophils and neutrophils; open circles represent CB and filled circles represent COPD.

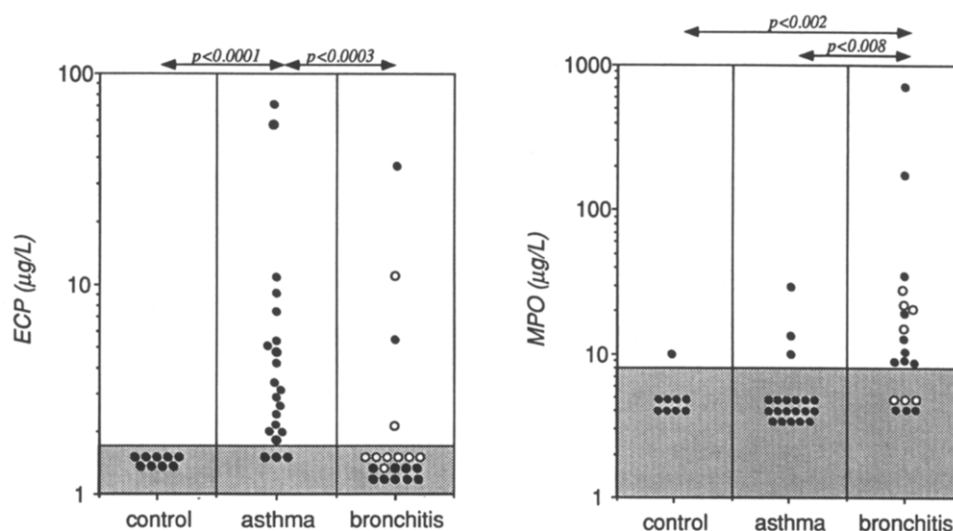


FIG. 3. Levels of ECP and neutrophil-specific MPO in BALF; open circles represent CB and filled circles represent COPD.

four groups for the enumeration of neutrophils as assessed by elastase monoclonal antibody staining: patients with CB, patients with asthma, and control subjects (Figs. 5 and 7). Although some of the neutrophils were degranulated in patients with CB or COPD, the degranulated score was not significantly increased in these patients in comparison with patients with asthma or normal subjects. In patients with CB, COPD, or asthma there was no significant correlation between the numbers of neutrophils in the biopsy specimens and MPO or the numbers of neutrophils in the BALF.

DISCUSSION

This research shows an increased number of eosinophils in the blood, BALF, and bronchial biopsy specimens of patients with asthma, CB, or COPD in comparison with normal subjects. However, only in the patients with asthma were eosinophils found to be degranulated. Neutrophils are not increased in bronchial biopsy specimens of patients with asthma, CB, or COPD; but in the BALF of patients with CB and COPD, they are both increased in number and activated as shown by the higher levels of MPO in BALF.

The diagnosis of some patients with airflow

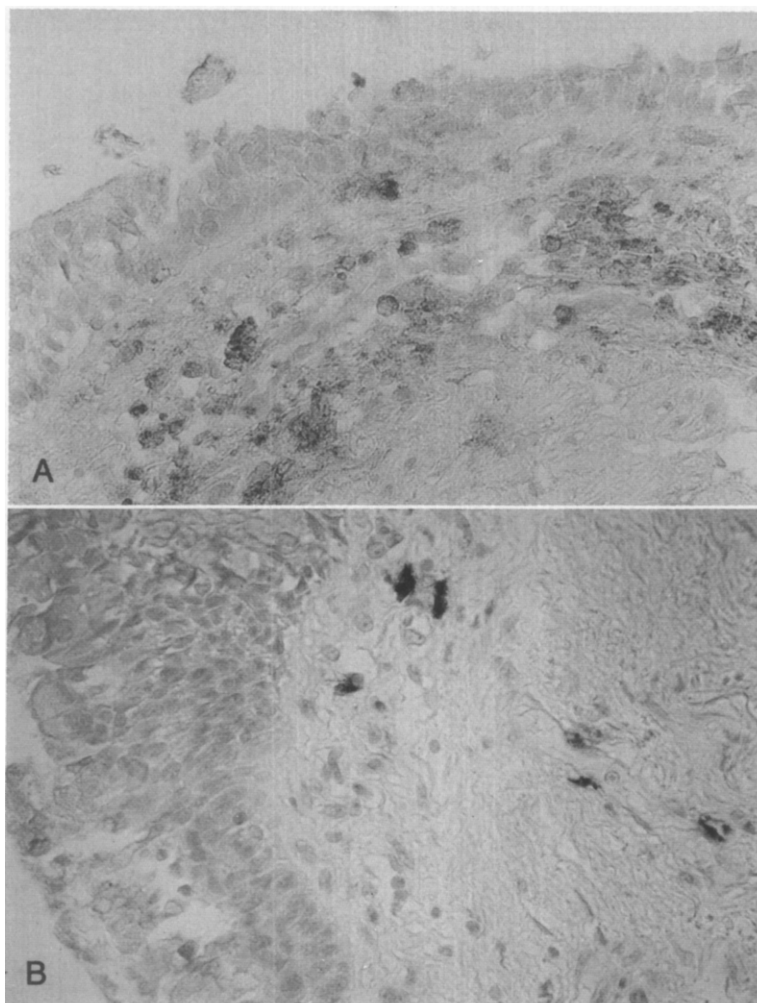


FIG. 4. **A**, Immunohistochemical analysis of degranulated eosinophils in a biopsy specimen of a patient with asthma ($\times 620$); **B**, immunohistochemical analysis of nondegranulated eosinophils in a biopsy specimen of a patient with CB ($\times 620$).

obstruction may be difficult¹; therefore, one of the major features of the present research was the careful characterization of patients with asthma, CB, and COPD. For this purpose, we carefully selected the three groups and excluded many patients in whom the diagnosis might have led to some confusion. CB is a disease of the central airways that relates to chronic cough and sputum production. This may or may not be associated with airway obstruction; but when obstruction is present, it is clearly related to the disease in the peripheral airways, particularly to the small bronchi and bronchioles. We diagnosed patients as having CB when there was no airway obstruction (FEV_1 over 80%); patients with COPD were those with an airway obstruction (FEV_1 under 70%). Interestingly, there was no patient with

FEV_1 that ranged from 70% to 80%. Moreover, patients with CB or COPD were excluded if they had a reversible airway disease or a possible allergic cause because they might have been diagnosed as having asthma. Moreover, we selected those patients who were smokers because this is one of the major causes of CB or COPD. On the other hand, we excluded from the study all patients with asthma who had smoked because we observed that these patients had increased levels of MPO in the BALF.¹⁹ Thus although the groups of patients selected do not represent the whole spectrum of the disease of asthma, CB, or COPD, the diagnosis was perfectly ascertained.

Many methods may be used to assess airway inflammation. On the basis of previous studies in asthma,^{4, 19, 20} we decided to use the combination

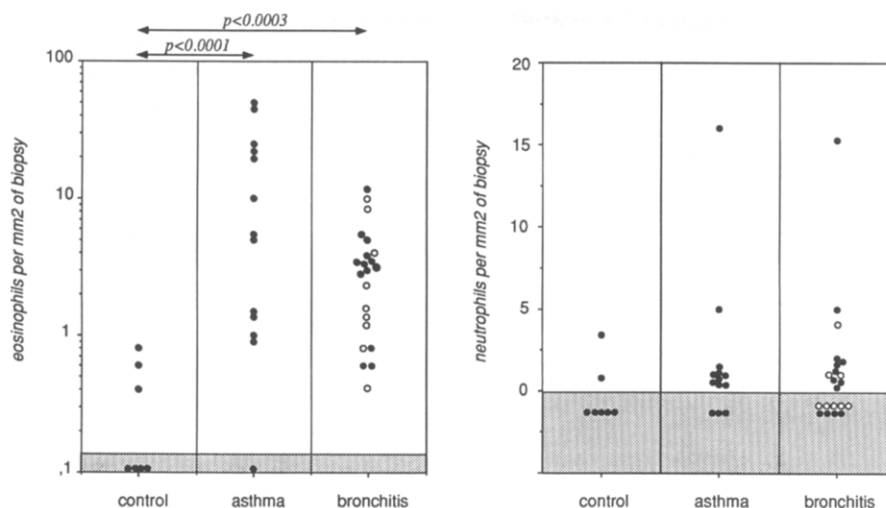


FIG. 5. Eosinophils (EG2 reactive cells) and neutrophils (anti-elastase reactive cells) in bronchial biopsy specimens; *open circles* represent CB and *filled circles* represent COPD.

of BALF analysis and immunohistochemistry in bronchial biopsy specimens. All drugs that might have modified the results of the study were excluded for an appropriate time, and in particular, the use of corticosteroids was carefully checked. β_2 -Agonists were only stopped for 8 hours before bronchoscopy because some patients with asthma had severe disease; however, it is unlikely that these drugs modify bronchial inflammation.⁴

Methods that make it possible to characterize eosinophil and neutrophil inflammation have been examined. We have previously studied eosinophil and neutrophil inflammation in asthma^{4, 19, 20} and found that the markers used in the present study were useful. EG2 monoclonal antibody reveals an epitope of ECP, and the results of biopsy and BALF analyses can be easily compared. Because eosinophils possess peroxidase, it was important to use a control that was always negative, indicating that the results of the present study can be interpreted without artifacts. Nondegranulated eosinophils were observed with HE staining; and the degranulation of eosinophils was easily observed, although a more precise visualization of this phenomenon might have been obtained if we had used BMK 13 monoclonal antibody²¹ or the carbol chromotrope 2R stain. Neutrophil MPO is also selective for neutrophils,¹³ but commercially available antibodies cannot be used for immunohistochemical studies on paraffin-embedded sections. We therefore studied MPO in the BALF and neutrophil-specific elastase in biopsy specimens, although the results obtained with these two markers may differ

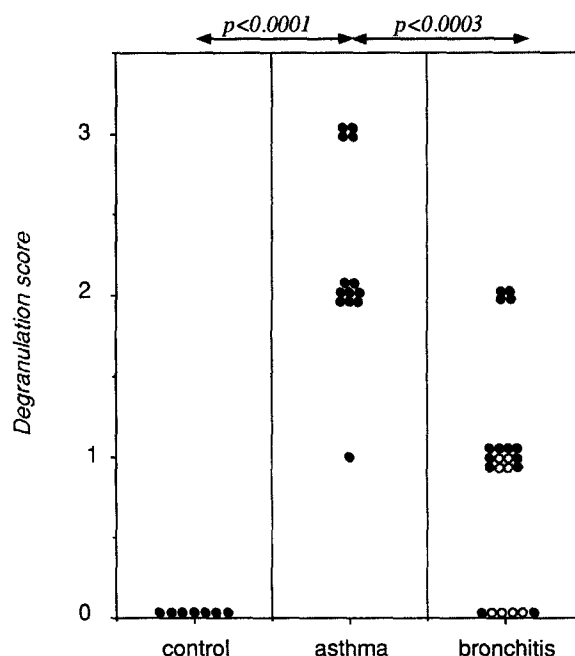


FIG. 6. Degranulation score of eosinophils in bronchial biopsy specimens; *open circles* represent CB and *filled circles* represent COPD.

slightly. We did not enumerate eosinophils and neutrophils in biopsy specimens by HE staining because degranulated cells cannot be characterized.⁴

The results of a specific marker in BALF may be expressed as its concentration in original "neat" BALF as micrograms per milliliter of fluid or calculated as a percent of total protein or as a ratio with albumin or urea.²² Although the BALF

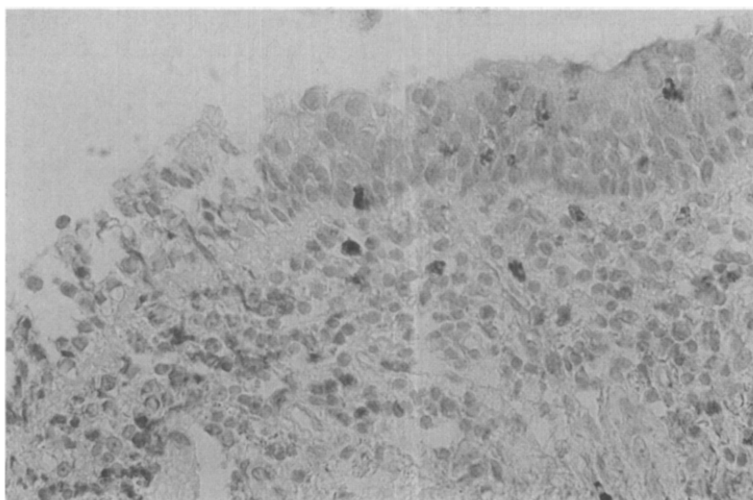


FIG. 7. Immunohistochemical analysis of nondegranulated neutrophils in a biopsy specimen of a patient with CB ($\times 620$).

recovery was different in patients and normal subjects, we expressed the results in micrograms per liter of BALF because the instilled volume was always the same and calculations of percentages or ratios do not therefore seem necessary²³ and because the choice of albumin or urea to express the results is not yet completely validated. Bronchial samples may be more related to bronchial or intraluminal inflammation, but we had to use alveolar samples only because trapping or acellular lavage fluid was observed in over 50% of the bronchial samples of patients with CB. Furthermore, it has been shown that the change in distal sample for the neutrophils in CB¹⁰ or for eosinophils in asthma tended to parallel the bronchial sample changes, but to a quantitatively lesser extent.

Eosinophilic inflammation in asthma is well known,^{3, 4} and the results of this study confirm those of previous experiments. Not only were the eosinophils increased in numbers in peripheral blood, BALF, and biopsy specimens, but as previously shown, they were degranulated and activated.⁴ There was a significant correlation between eosinophils or ECP in the BALF and the severity of asthma as previously reported,^{4, 19} but there was no correlation between eosinophil numbers in the biopsy specimens and the severity of asthma. On the other hand, the presence of eosinophilic inflammation in CB or COPD is more surprising but not completely unexpected. Increased numbers of eosinophils have been found in some cases: (1) in the peripheral blood of smokers or patients with CB,²⁴ (2) in the BALF

of patients with CB,¹¹ and (3) in bronchial post mortem biopsy specimens.^{7, 25} Postmortem specimens may, however, be difficult to evaluate because the medical history of the patient is not always completely characterized, and with conventional staining, eosinophils were not enumerated in biopsy specimens of patients with CB, which were obtained by fiberoptic bronchoscopy.²⁶

On the other hand, eosinophils were seldom found in induced sputum of patients with CB.²⁷ Thus this study is the first, to our knowledge, to demonstrate an increased number of eosinophils in biopsy specimens of living patients with CB or COPD. There was no difference in the numbers of eosinophils in the BALF or biopsy specimens and in the severity of the airway obstruction. Another original finding of the present study is that these eosinophils are seldom degranulated in these biopsy specimens and this is in striking contrast to the findings in patients with asthma. Moreover, ECP levels in the BALF are not increased in CB or COPD. In asthma, we previously showed that there was a significant correlation between ECP levels and eosinophil numbers in the BALF and FEV₁,^{4, 19} but in the present study, there is no such correlation in patients with CB or COPD. This study shows, however, that eosinophilic inflammation of the airways is not restricted to asthma. Combining the observations made in asthma and CB or COPD, it appears that in both diseases there is a chemoattractant signal for eosinophil migration toward the airways, but the activating signal mainly exists in asthma. It is tempting to speculate that the cytokine pattern,

possibly interleukin-5,²⁸ may differ in both diseases.

Neutrophils in the bronchial walls were rare in normal subjects, and we found no significant difference in the three groups of patients. In asthma, the contribution of neutrophils is unclear, and results of previous studies fail to show a difference between patients with asthma and control subjects for the neutrophilic inflammation.^{19, 20, 29} After challenge with isocyanate, a marked rise in the number of neutrophils occurs in BAL, but this was not confirmed in the bronchial biopsy studies.³⁰ In CB and COPD, the inflammatory cells infiltrating the bronchi consisted mainly of mononuclear cells such as lymphocytes²⁶ and occasionally neutrophils.^{8, 9} This study shows that neutrophils are not present in high numbers in bronchial biopsy specimens of patients with CB or COPD. This finding contrasts sharply with BALF in which large amounts of neutrophils are often recovered.^{10, 11} Smoking increases neutrophils in BALF but seems to have little or no effect on bronchial infiltration.^{31, 32} These observations may be explained by the study of Ludwig et al.,³³ which clearly demonstrated that cigarette smoking causes neutrophil accumulation in alveolar septum but not in the bronchi. Alternatively, these findings suggest that in CB or COPD the inflammatory component is located in the peripheral airways rather than in the central airways. The neutrophils are likely to be activated in the BALF of patients with CB because a high amount of MPO was detected. This activation is less evident in the bronchi, although in a few patients neutrophil degranulation was identified with the use of anti-elastase antibody. This neutrophil recruitment in the BALF might be due to neutrophil-chemotactic factors released by airway cells such as alveolar macrophages, T lymphocytes, and perhaps epithelial cells.³⁴ The relevance of neutrophils found in the airway lumen of patients with CB is not completely understood, but previously shown by Martin et al.¹⁰ and Thompson et al.,¹¹ we found a correlation between neutrophil numbers in BALF and FEV₁. The increased number of neutrophils may increase elastolytic activity of BALF and in turn participate in the decrease of the elastic recoil.³⁵

Although recent immunocytochemical studies of the peripheral airways have shown that the number of neutrophils present in the peripheral airways correlates with the amount smoked but not with airway obstruction,³² we were unable to observe any significant correlation between the

number of pack-years smoked and any of the parameters studied. This may be due to the difference in sampling the airways because we only examined large airways, and tobacco smoke may preferentially affect small airways. Alternatively, the lack of correlation between smoking and airway inflammation may be linked to the difficulties in correctly assessing smoke inhalation over very long periods of time and in the variation of smoke inhalation during the smoking period; some patients with severe COPD tend to decrease smoking, although they do not completely stop (in the present study). It has been shown by Rennard et al.³⁶ that short-term smoking reduction is associated with reduction in inflammation assessed by BAL (and particularly neutrophils).

Finally, this research may explain the inconsistent effect of corticosteroids in the treatment of CB.^{37, 38} The influence of patient characteristics on the response to corticosteroids has been previously examined. It has been suggested that patients with the most severe airflow obstruction were those with the least improvement. However, factors other than baseline FEV₁ may predict corticosteroid response in groups and in individual patients. A higher peripheral blood eosinophil count has been found as a predictor of response in some but not all studies.^{37, 38} Peripheral blood eosinophils may be less accurate than bronchial eosinophils, but proper studies should be carried out to confirm this hypothesis.

This research shows the difference and the relative specificity of granulocyte infiltration and activation in patients with asthma and CB. It is suggested that granulocytes might be margined in different parts of the lung: eosinophils in the bronchi of patients with asthma and neutrophils in alveoli of patients with CB. These findings might be relevant to the therapeutic strategy for both diseases.

We thank Dr. Harry Clark for reviewing the manuscript.

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