Neutrophils infiltrating bronchial epithelium in chronic obstructive pulmonary disease

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In order to characterize neutrophil and eosinophil presence in the airways of patients with chronic obstructive pulmonary disease (COPD), bronchoscopy with bronchial washings and bronchial biopsies was performed in 12 smoking stable COPD subjects and 18 normal non-smoking control subjects. Bronchial biopsies were examined by light microscopy using plastic embedding and histochemical techniques to identify different cell types.

Bronchial washing fluid of COPD patients was characterized by a predominance of neutrophils ($P=0.001$), and a slight, but significant ($P=0.03$), increase of eosinophil fraction. Subjects with COPD had higher number of neutrophils in the epithelium ($P=0.01$), and eosinophils in the lamina propria ($P=0.01$) than did control subjects. The thickness of reticular basement membrane was increased for COPD patients in comparison to control subjects ($P=0.01$).

The present study provides evidence of neutrophil infiltration both in bronchial washing and bronchial epithelium of patients with COPD, suggesting that the source of neutrophils in airway lumen may be the bronchial mucosa. Although less common than in asthma, airways of COPD subjects reveal eosinophil presence and airway remodelling.

Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a condition characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema; the airflow obstruction is generally progressive, may be accompanied by airway hyperreactivity, and may be partially reversible (1). The pathological findings of COPD are characterized by goblet cell metaplasia, submucosal gland hypertrophy, and airway wall fibrosis and inflammation (2). The characteristics of the inflammatory reaction are different in bronchial tissue and at the epithelial surface of the airways: activated macrophages and T lymphocytes, predominantly of the CD8 phenotype (3), together with mast cells, predominate in bronchial mucosa, in the interstitium and periglandular areas (4–10), while polymorphonuclear leukocytes have been found in increased proportions in the airway secretions (11–13) and in lavage fluid (14–16).

Although current knowledge on the pathogenesis of this disorder does not provide convincing explanations for this discrepancy, it is clear that neutrophils can play a major role in inducing progressive airway damage.

Studies which failed to show an increased number of neutrophils in bronchial mucosa of COPD patients (9,17–20) used immunocytochemical methods to identify neutrophils. Immunohistochemistry is the most sensitive methodology to identify cells in tissue samples, however, the monoclonal antibodies available for neutrophil identification have low specificity (21,22). For this reason, in this study as in previous studies (23,24), we used a simple histological technique to identify different cell types.

In the present study, in order to characterize neutrophil and eosinophil presence in the airways of patients with COPD, bronchoscopy with bronchial washings and bronchial biopsies was performed in 12 smoking stable COPD subjects and 18 normal non-smoking control subjects.

Materials and Methods

SUBJECTS

Twelve smoking patients with COPD (11 males and one female, mean age 62.6 ± 2.0 years) and 18 normal non-smoking subjects (13 males and five females, mean age

59·4 ± 3·2 years) were included in the study. Subjects with COPD were characterized by cough and mucoid sputum production on most days of the month, for at least 3 months per year during at least the previous 2 yr. The subjects met the following criteria: (1) no personal or family history of allergic disease; (2) no exacerbation of their disease, defined as increased dyspnoea associated with changes in quality (mucoid to purulent) and quantity of sputum within the preceding month; (3) no therapy with oral or inhaled glucocorticoids or inhaled cromolyn within 3 months before entering the study; (4) forced expiratory volume in 1 s (FEV1) ≤75% of predicted values, (5) no improvement in FEV1 of over 15% after inhalation of 200 μg of salbutamol, and (6) no history of systemic disease or of other pulmonary disease (patients with obvious emphysema, as assessed by routine chest X-ray studies were also excluded). Subjects were not excluded if they were receiving theophylline, β-agonists or both.

All subjects underwent spirometric function tests 1 day before bronchoscopy (Spiroflow, Morgan, Kent, U.K). Blood eosinophils were counted on the same day as the pulmonary function tests. Their smoking history was carefully checked, and a minimum of 15 pack-years smoked was required (16·5–80 pack-years, mean ± SE: 38·6 ± 6·7 pack-years).

The normal age-matched volunteers were asymptomatic lifelong non-smokers, without clinical or laboratory signs of sensitization to common allergens and normal spirometric values. The volunteers were divided in two groups, the first group (ten subjects) performed only bronchial washing (BW), the second (eight subjects) only the bronchial biopsies. The study protocol had the approval of the local ethics committee, and each person gave informed written consent before undergoing the bronchoscopic procedures.

BRONCHOSCOPY PROCEDURES

Bronchoscopy was performed between 8 and 9 a.m. on each occasion, according to National Institutes of Health Guidelines (25). Subjects abstained from food for at least 12 h before bronchoscopy. Premedication consisted of nebulized salbutamol (1·25 mg) and ipratropium bromide (0·5 mg), atropine (0·5 mg) by i.m. injection administered 30 min. before the procedure. Upper airways were anaesthetized by 4 ml of lignocaine at 2%. Further lignocaine (1%) was administered, after flexible fibreoptic bronchoscope (1T10 Olympus, Tokyo, Japan) introduction, to the lower airways to suppress coughing. The bronchoscope was wedged into a segment of the right middle lobe, and one 50-ml aliquot of sterile saline solution, warmed at 37°C, was infused. Fluid was gently aspirated immediately after the aliquot was introduced and collected in a sterile container (BW). This is corresponding to the first lavage sample of a bronchoalveolar lavage (BAL) which is considered more reflective of the content of conducting airways, whereas the further samples are more related to bronchioles and alveoli (26).

Three bronchial specimens were taken with alligator forceps at the subcarinal level in the lower and middle lobes of the right lung.

During the procedure, oximetry using a fingerprobe (Pulse Oxymeter 504, Critical Care System Inc., U.S.A.) and electrocardiograph (ECG) tracing were continuously monitored. Supplemental oxygen was delivered continuously via a nasal cannula at a rate of 3 l min⁻¹.

HANDLING OF BW FLUID

After recovery, BW fluid was strained through a monolayer of surgical gauze to remove mucus. The fluid was immediately centrifuged at 800 rpm for 10 min. Supernatant was removed and the cell pellet was washed twice with phosphate-buffered saline (PBS) solution (without Ca^{2+} and Mg^{2+}). Cytocentrifugates (Labofuge AE, Heraeus, Germany) were stained by the May–Grunwald–Giemsa method. The differential cell count of macrophages, lymphocytes, neutrophils, and eosinophils was made under light microscopy at × 1000 (BH-2, Olympus), by counting approximately 300 cells in random fields. Results were expressed as percentage of total cells and as total number.

HANDLING OF BRONCHIAL BIOPSY SPECIMENS

The bronchial biopsy specimens were immediately fixed in periodate–lysine–paraformaldehyde (pH 7·4) for 3 h and then washed in a buffered cacodylate solution with 7% sucrose for 12 h. Each specimen was embedded in glycol methacrylate (JB4 Polysciences Inc., Newton, MA, U.S.A.).

Tissue sections, 2 μm thickness, were stained with haematoxylin–eosin and toluidine blue and examined under light microscopy at × 1000 magnification (S plan, Olympus). Two sections at 100 μm intervals from each biopsy were selected and appropriately stained for each quantitation to avoid cell overlapping.

Neutrophils, identified by their characteristic nucleus (multilobulated and hyperchromic without nucleoli) staining with toluidine blue (Fig. 1), eosinophils, identified by their eosinophilic staining, were then counted in the intact epithelium (as defined by the presence of both basal and columnar cells, with no appearance of metaplasia), and in the lamina propria (it was arbitrarily taken as a zone 80 μm deep to the limit of the reticular basement membrane, as defined by a squared eyepiece graticule).

The thickness of the reticular basement membrane was assessed on the two sections stained with haematoxylin–eosin and was measured from the base of the bronchial epithelium to the outer limit of its reticular basement membrane at regular 200-μm intervals along the length of each section, using a graduated eyepiece graticule. The final result was a single value per patient obtained from the averages of all measurements performed in two sections from each biopsy. The stained sections were coded and examined by a single-blinded investigator (A.P.). The cells were counted in adjacent non-overlapping high-power fields until all the available area was covered.

The final result was a single value per patient obtained from the average of the measurements performed for each section. The cell counts were expressed as number of cells

TABLE 1. Characteristics of patients with COPD and 18 healthy control subjects

Subject no.	Age (years)	Sex	Atopy	Smoke (pack-yr)	Blood eosinophils $n \mu l^{-1}$	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	FEV1 (% pred)	FEV1/FVC (%)
COPD patients									
1	69	M	No	50	120	72·2	44·7	71	66
2	57	M	No	17·5	410	78·4	34·8	75	70
3	69	M	No	55	40	70	44·6	74	69
4	68	M	No	50	170	65·1	45	74	68
5	55	M	No	40	170	68	38·2	75	71
6	69	M	No	40	330	66·6	46·3	70	70
7	69	M	No	51	170	65·4	49·8	75	68
8	67	M	No	50	240	77	43·8	73	64
9	57	M	No	80	140	78·9	34·5	60	57
10	48	M	No	16·5	120	71·6	40·5	61	58
11	63	M	No	24	80	65·3	46	70	60
12	61	F	No	24	60	72	45·3	75	65
x mean	62·6	11M/1F	—	38·6	170·8	70·8	42·7	71	65·5
SE	2			6·7	31·5	1·4	1·3	1·5	1·4
Healthy control subjects									
x mean	59·4	13M/5F	No	No	148·7	95·5	39·6	103·1	85
SE	3·2				28·8	3·8	1·1	8·2	2

per millimetre length of intact epithelium for the epithelial infiltrate, and as number of cells per square millimetre for the lamina propria. Microphotographs were taken using a 35-mm microscope camera (PM-6, Olympus), and film (Kodak Ektachrome 64T professional) was used with semi-automatic controlled exposure times (EMM-7, Olympus).

DATA ANALYSIS

Group data were expressed as medians and range, or means ± SE when appropriate. Comparisons between biopsy data of bronchitic and control subjects were made using the Mann–Whitney U test; Spearman rank test was used to examine the association of histological parameters with BW and physiological data; we accepted a P value <0·05 as indicating significance. The coefficient of repeatability, as described by Bland and Altman (27), was used to compare measurements performed on the two sections of the same biopsy. At least three replicate measurements of morphometric parameters were performed by the same observer, and the intra-observer reproducibility was assessed with the coefficient of variation for repeated measurements.

Results

Bronchoscopy with BW and endobronchial biopsies were successfully performed and well tolerated in all subjects.

CLINICAL FINDINGS

The demographic characteristics of the subjects are presented in Table 1. The mean age of patients with COPD and normal subjects was similar. The pulmonary function of normal subjects was within the normal range. All the patients with COPD had the FEV1 ≤75% predicted, and the FEV1 did not improve significantly after salbutamol inhalation. The total number of peripheral blood eosinophils was similar for COPD and control subjects. All subjects were non-atopic, i.e. they had negative skin tests for common allergen extracts and no personal or family history of allergic disease.

BRONCHIAL WASHING

The number of cells and the differential counts in normal subjects and in patients with COPD are shown in Table 2.

Neutrophils and eosinophils were significantly increased in patients with COPD in comparison with normal subjects. On the other hand, macrophages and lymphocytes were significantly reduced in COPD with respect to control subjects. Percentages of epithelial cells were similar in the COPD group compared with the group of controls (19·5 ± 7·3 vs 13·2 ± 6·4 n.s., respectively). In patients with COPD there was no correlation between smoking and any of these counts.

BIOPSY FINDINGS

Resin embedding of tissue permitted sections to be cut much thinner (1–2 μm) than paraffin sections, allowing

TABLE 2. Bronchial washing in COPD and control subjects

	Macrophages		Lymphocytes		Neutrophils		Eosinophils	
	%	no. $\times 10^3$	%	no. $\times 10^3$	%	no. $\times 10^3$	%	no. $\times 10^3$
COPD patients								
Median	32·45	21·1	1	0·75	63·2	62·6	1	1·6
Range	2·2–96·9	2·1–207·5	0–4·3	0–5·6	1·9–94·5	1·2–323	0–19·8	0–6·9
Healthy control subjects								
Median	91·65	101	2·65	3·9	1·4	1·35	0·2	0·15
Range	79·6–100	63–130	0–12·6	0–18·8	0–12·8	0–19·2	0–1·1	0–3·7
P	0·001	0·012	0·041	0·027	0·001	0·001	0·032	0·035

better optical definition of tissue and cell structure. Neutrophils (Fig. 1) and eosinophils were easily identified in specimens of bronchial tissue.

The coefficients of repeatability between measurements performed on the two sections of each biopsy were 0·76 neutrophils, and 0·45 eosinophils mm $^{-1}$ for the epithelium, and 28·5 neutrophils, and 13·8 eosinophils mm $^{-2}$ for lamina propria. The mean coefficient of variation for three repeat cell counts by the same observer (A.P.) ranged from 5% to 16% for the cells studied. The coefficient of variation of the assessment of the thickening of reticular basement membrane was 4%.

Cell counts in biopsy specimens of COPD patients and control subjects are summarized in Table 3.

Patients with COPD had an increased number of neutrophils in epithelium (Fig. 2) when compared with control subjects, whereas the number of eosinophils was increased in the lamina propria (Fig. 3). The thickness of the reticular basement membrane, as assessed by light microscopy, was increased for COPD patients in comparison to control subjects ($P<0·01$) (Fig. 4).

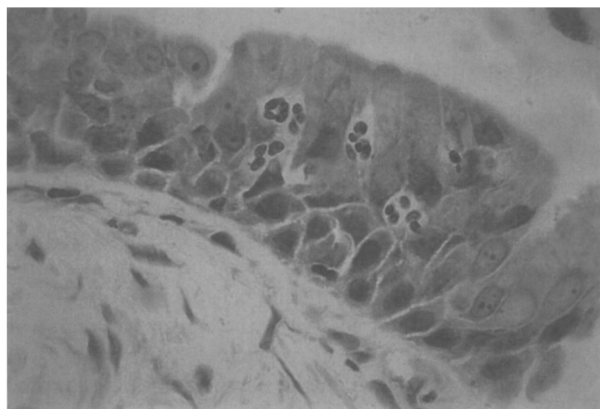No correlations were found among data.

FIG. 1. Light photomicrograph of several neutrophils in the bronchial epithelium, from a patient with COPD. Neutrophils are easily recognised by their multilobulated and hyperchromic nucleus (toluidine blue, original magnification: × 400).

Discussion

This study shows an increased number of neutrophils in the epithelium of living patients with COPD in comparison to normal healthy control subjects. In addition, an increased number of eosinophils in the lamina propria and thickness of the reticular basement membrane in the bronchial mucosa of the group of COPD patients are observed.

Previous studies evaluating the inflammatory changes have demonstrated that the principal airway lumen change in patients with COPD, is an increase in the number of neutrophils (11–16), while there is a predominance of mononuclear cell infiltration in the mucosa (4–9). This discrepancy has given rise to the hypothesis that the luminal and the parenchymal inflammation may be different. Our observation permits us to hypothesize that the source of neutrophils in airway lumen of COPD patients is the bronchial mucosa and that lavage findings reflect the pathology of airway mucosa, and in particular its epithelium. This confirms recent findings published in preliminary form (20).

It can be argued that a larger percentage of neutrophils was observed in bronchial lumen than was observed in bronchial mucosa of our COPD patients, and that no correlation was found between the percentage of neutrophils in BW and that in bronchial mucosa. However, it may be unreasonable to expect that the findings of sputum, bronchial washing, and bronchoalveolar lavage would closely mimic those of biopsies of COPD patients given the dynamic nature of the inflammatory response and the differences in cell populations found on either side of the epithelial basement membrane of the normal lung. Static histopathological examination of migratory cells such as neutrophils and eosinophils would be expected to show only those cells presently in transit between the blood and the bronchial lumen, whereas lavage fluids would better reflect the magnitude of cell migration into the bronchus over time. There is every reason to think that histopathological and lavage fluid findings will be complementary and mutually informative in studying bronchial disorders.

The increased number of neutrophils in the epithelium and the absence of any statistical difference in their number in the lamina propria of COPD patients in comparison to

TABLE 3. Endobronchial biopsy in COPD and control subjects

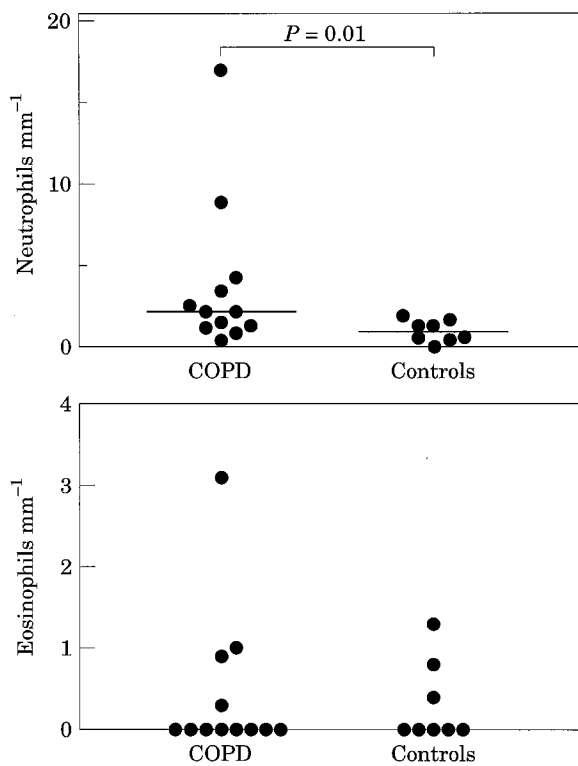|	RBM μm	Epithelium (cells mm^{-1})		Lamina P (cells mm^{-2})	
		Neutrophils	Eosinophils	Neutrophils	Eosinophils
COPD patients					
Median	6	2·15	0	39·2	26·6
Range	3·6–8·6	0·4–16·9	0–3·1	12·7–149·3	9·8–60·3
Healthy control subjects					
Median	4·3	0·9	0	22·85	4·45
Range	3·8–5·2	0–1·9	0–1·3	7·6–59·2	0·33
P	0·01	0·01	n.s.	n.s.	0·01

RBM, reticular basement membrane; Lamina P, lamina propria.

FIG. 2. Neutrophils and eosinophils in the epithelium of control and COPD subjects. Horizontal bars represent group median values.

FIG. 3. Neutrophils and eosinophils in the lamina propria of control and COPD subjects. Horizontal bars represent group median values.

control subjects could sustain the hypothesis that epithelium-derived factors linked to the disease state are crucial to the retention of neutrophils within the epithelium. In this regard, interleukin-8 (IL-8) a potent neutrophil chemoattractant and activator, which can be produced by epithelial cells (28), was found increased in sputum (13) and bronchial washing (29) in COPD.

The concentration of neutrophils in BAL fluid has been reported to be negatively correlated with the FEV1 (14–16), and positively with smoking history (14,16,19). We were unable to observe any significant correlation between neutrophil counts and the number of pack-years smoked, or FEV1. This may be due to the relatively small number of
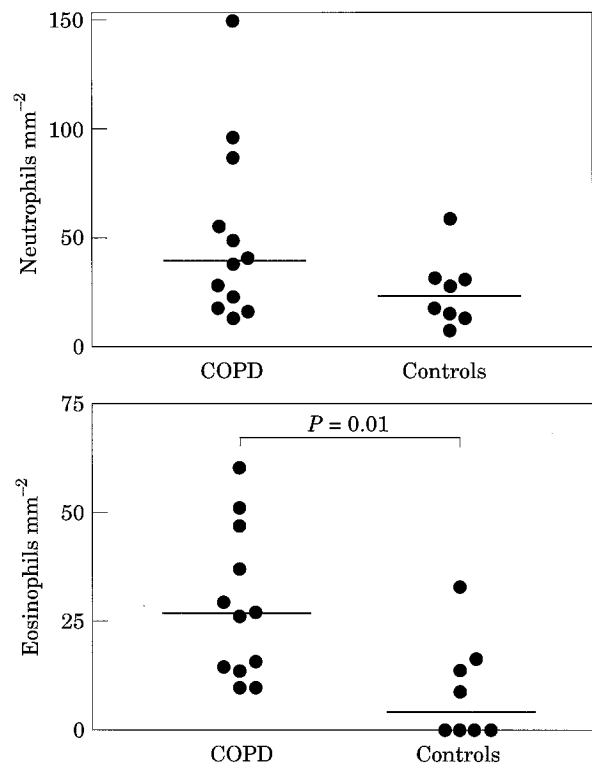
subjects that we examined, the different selection criteria of patients, the difference in airway sampling, and to the difficulties in correctly assessing the numbers of cigarettes smoked over very long periods of time. In any case, larger populations of COPD and control smoking subjects may be required to clarify associations between neutrophil inflammation, smoking history, and airway obstruction. Previous studies examining the percentages of macrophages and lymphocytes in bronchial lumen of COPD patients reported conflicting results (see 10,14,16 and 17). The reduced percentages of macrophages and lymphocytes in bronchial washing of our COPD patients may be explained by the elevation of neutrophils in the same compartment. The
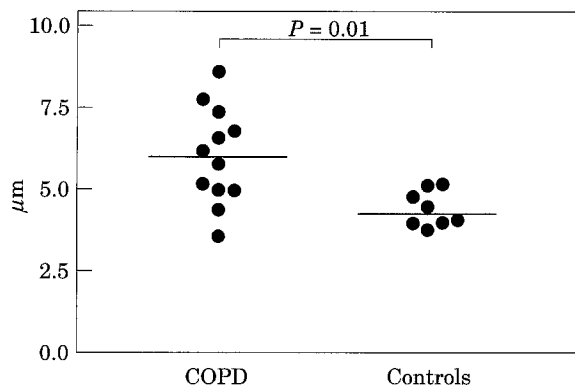
FIG. 4. Thickness of reticular basement membrane of control and COPD subjects. Horizontal bars represent group median values.

mechanism that causes neutrophil accumulation remains speculative, but some possibilities can be postulated. Recently, increased expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and E-selectin (30) in the endothelium has been shown on bronchial biopsy of patients with COPD, and tumour necrosis factor-a, a cytokine known to upregulate adhesion molecules, has been demonstrated to be elevated in induced sputum of patients with COPD in addition to the potent neutrophil attractant and activator IL-8 (13).

The eosinophil findings support previous observations (16,17,31) and confirm that some eosinophils are present in the airways of the subjects with stable COPD, although this is far less common than in asthma (32,33). Eosinophil-directed haematopoietins, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-3, or interferon-γ produced by T-lymphocytes may increase eosinophils in tissues by prolonging their survival (34,35). This mechanism is conceivable in chronic bronchitis where activated lymphocytes in bronchial mucosa were demonstrated (9). Moreover, recent studies (31,36) demonstrated an effect of bronchial eosinophilia on airflow obstruction in patients with COPD. In particular, Nagai et al. (36) showed that in COPD patients flow rates are adversely affected by bronchial eosinophilia. Chanez et al. (31) demonstrated that corticosteroid reversibility in COPD patients is related to the presence of eosinophilic inflammation in the airways of these subjects.

Reticular basement membrane thickness in COPD patients was increased, but to a lesser extent than in asthmatic subjects analysed with the same methodology (37). Our data confirm the observations of Lacoste et al. (17) who showed an increase of reticular basement membrane thickness in most patients with chronic obstructive pulmonary disease, and differ from those of Saetta et al. (9), Ollerenshaw et al. (38), and Trevisani et al. (39) who did not observe significant differences of reticular basement membrane size in comparison to control subjects. The different findings of these studies may be explained by the different populations (different degrees of obstruction, different disease duration) of chronic bronchitis patients evaluated or by different methodology used. The thickness of reticular

basement membrane in our COPD patients is similar to that of control subjects in most of the reports of the literature, this may be explained by the fact that in the present study bronchial biopsy specimens were embedded in glycol methacrylate whereas in the other studies paraffin embedding has been used, which is known to cause shrinkage of the tissue samples.

It is reasonable to postulate that the thickness of reticular basement membrane in COPD could contribute to airway obstruction. In fact, airway obstruction in chronic bronchitis has a multifactorial pathogenesis and is linked not only to acute inflammatory events like tissue oedema, and cell infiltration, but also to irreversible end-stage lesions such as a connective tissue deposition, and distortion of the membranous bronchioles by loss of elastic recoil (40).

Our observations of an increased number of eosinophils and thickness of reticular basement membrane in bronchial mucosa of COPD patients nicely fit with the 'Dutch hypothesis', that considers asthma and COPD to be different expressions of the same disease. In any case, larger populations of COPD patients may be required to establish if the presence of a thick reticular basement membrane and eosinophils in biopsy sections may differentiate asthma from COPD.

In conclusion, the present study provides evidence for neutrophil and eosinophil infiltration in bronchial mucosa of COPD patients, supporting the involvement of these cells in the pathogenesis of the disease.

Acknowledgement

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