Bronchial inflammation in chronic bronchitis assessed by measurement of cell products in bronchial lavage fluid

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Abstract

Background – **Bronchial inflammation in chronic bronchitis has not been characterised as well as in asthma. The present study was undertaken to assess whether a characteristic pattern of bronchial inflammatory markers could be found in patients with chronic bronchitis.**

Methods – **Bronchoscopy with bronchial lavage was performed in 42 patients with chronic bronchitis and in 13 healthy controls. Twenty three of the patients had non-obstructive chronic bronchitis and 19 had chronic bronchitis and chronic obstructive pulmonary disease (COPD). Eighteen of the patients with bronchitis had recurrent infective exacerbations and 24 did not. Intrabronchial bacterial cultures were taken with a protected specimen brush.**

Results – **Increased activity of neutrophils, fibroblasts, and eosinophils was found in the patients with chronic bronchitis as assessed by the levels of myeloperoxidase (MPO) and interleukin-8 (IL-8), hyaluronan, and eosinophil cationic protein (ECP), respectively. The levels of tryptase did not differ from the controls. High correlations were found between the levels of MPO and IL-8, as well as ECP and IL-8. No differences were found between the patients with COPD and those with non-obstructive chronic bronchitis.**

Conclusions – **Recruitment and activation of both neutrophils and eosinophils seem to be a characteristic of chronic bronchitis. This activation is associated with IL-8. The patients with intrabronchial cultures of *Streptococcus pneumoniae* had the highest individual levels of MPO, ECP, and IL-8 of all subjects in the study, indicating that colonisation with *S pneumoniae* could promote bronchial inflammation.**

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Keywords: chronic bronchitis, chronic obstructive pulmonary disease, airways inflammation, eosinophils, neutrophils.

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Chronic bronchitis is a clinical entity characterised by cough and mucus hypersecretion. It can be associated with bronchial obstruction (chronic obstructive pulmonary disease, COPD) and recurring infective exacerbations.[1] The pathological changes in the respiratory tract consist of goblet cell hyperplasia and submucosal gland hypertrophy, as well as bronchial inflammation.[2 3]

The airways inflammation in chronic bronchitis has not been characterised as well as in bronchial asthma. Histopathological studies of large airways mucosa in patients with chronic bronchitis have demonstrated infiltration of mononuclear phagocytes,[4] lymphocyte infiltration, and lymphocyte activation.[5] In contrast, mucosal changes in asthmatic patients have shown a predominance of eosinophils and mast cells.[6–9]

Bronchoalveolar lavage (BAL) fluid, as well as bronchial lavage fluid, from patients with chronic bronchitis has shown raised levels of macrophages and neutrophil granulocytes.[10 11] In lavage fluid from patients with chronic bronchitis one would therefore expect to find products from activated neutrophil granulocytes such as myeloperoxidase (MPO), and chemotactic factors for neutrophil granulocytes such as interleukin 8 (IL-8). In addition, since high levels of the acute phase reactant interleukin 6 (IL-6) have been found in the urine of patients with asymptomatic bacteriuria,[12] this could also be a potential marker of local bacterial colonisation and bronchial mucosal inflammation in lavage fluid from patients with chronic bronchitis.

We wished to study whether the airways inflammation in smokers with chronic bronchitis is reflected in raised levels of inflammatory markers in bronchial lavage fluid compared with healthy non-smoking controls. The second aim was to determine whether levels of inflammatory markers differed between patients with non-obstructive chronic bronchitis and patients with chronic bronchitis and COPD, as well as between patients with chronic bronchitis and recurrent infective exacerbations and those without. The third objective was to assess whether the levels of markers correlated with the intrabronchial bacterial flora in the patient groups.

Methods

STUDY DESIGN

Small volume lavage of the large airways was used, a method known to give samples predominantly representative of the bronchial epithelium.[13–15] The presence of MPO, tryptase, hyaluronan, and eosinophil cationic protein (ECP) were used as indirect markers for activation of neutrophils, mast cells, fibroblasts and eosinophils, respectively, and IL-6 and IL-8 were used to indicate a broader activation of cells including epithelial cells, granulocytes, and lymphocytes. All patients were investigated during an infection-free period.

Table 1 Mean (range) age, sex, and mean (SE), FEV₁ (% of predicted normal) and cigarette consumption (pack years) of healthy non-smoking controls and patients with chronic bronchitis

Subjects		n	Age (years)	FEV₁	Pack years
A. Healthy controls (4M/9F)		13	47 (26–66)	101 (1)	0
B. Chronic bronchitis (22M/20F)		42	55 (36–70)	79 (3)***	40 (2)
B(1a)	Non-obstructive chronic bronchitis (10M/13F)	23	52 (36–38)	93 (2)	37 (2)
B(1b)	Chronic bronchitis with COPD (12M/7F)	19	57 (38–70)	63 (2)**	44 (4)
B(2a)	Chronic bronchitis without infective exacerbations (16M/8F)	24	55 (36–70)	81 (4)**	40 (3)
B(2b)	Chronic bronchitis with infective exacerbations (6M/12F)	18	54 (38–68)	77 (4)**	40 (4)

** $p < 0.01$, *** $p < 0.001$ v controls (Mann-Whitney U test with Bonferroni's correction for multiple comparisons).

SUBJECTS

Thirteen healthy lifelong non-smoking volunteers with normal spirometric values and no signs of infective respiratory disease during the past four weeks comprised a control group (group A). They were compared with 42 patients with chronic bronchitis (group B), all of whom were current smokers with a productive daily cough for at least three consecutive months each year for the past two years. These patients were subdivided into two groups according to the presence or absence of airways obstruction: group B (1a) – 23 patients with non-obstructive chronic bronchitis and normal spirometric values, and group B (1b) – 19 patients with chronic bronchitis and COPD (defined as a forced expiratory volume in one second (FEV₁) of less than 80% of predicted and a reversibility less than 10% on β₂ agonists). They could also be subdivided into two groups according to the presence or absence of infective exacerbations: group B (2a) – 24 patients without a history of recurrent infective exacerbations, and group B (2b) – 18 patients with three or more antibiotic treated infective exacerbations during the past two years as defined by Boman et al.[16]

The patients with chronic bronchitis were slightly older and had a lower mean maximum FEV₁ (percentage predicted normal) than the healthy controls. The smoking history (in pack years) and age between subgroups of patients with chronic bronchitis were comparable (table 1).

Of the 23 patients with non-obstructive chronic bronchitis, none used oral N-acetylcysteine or inhaled medication on a regular basis. Of the 19 patients with chronic bronchitis and COPD, eight used oral N-acetylcysteine regularly (200 mg at least twice daily) and four used inhaled corticosteroids regularly. No patients used theophylline preparations or oral steroids.

The duration of symptoms in the patient group varied from 2·5 years to 24 years with a mean of 12·5 years. Criteria for exclusion were abnormal chest radiography, bronchial hypersecretion caused by factors other than cigarette smoking, a history of asthma or reversibility >10% after β₂ agonists, and for safety reasons an FEV₁ less than 50% predicted or age more than 70 years. Ventilatory function (FEV₁) was measured with a Bernstein spirometer or Vitalograph.

The study design was approved by the ethical committee of the University of Göteborg and the volunteers gave their consent after both written and oral information.

FIBREOPTIC BRONCHOSCOPY
Premedication was given with haloperidol 5 mg orally followed by 0·5–1 ml morphine-scopolamine intramuscularly. Five ml 1% tetracaine without preservative was nebulised (Plug-in inhalator, Aiolos Systems, Karlstad, Sweden) and inhaled in an upright position for local anaesthesia. All bronchoscopies were performed transorally by one of two experienced bronchoscopists, and with the patients in the supine position. Several models of Olympus flexible fiberoptic bronchoscopes were used.

Bacterial samples were taken during bronchoscopy according to the method of Wimberley et al using a telescoping double catheter protected specimen brush (Microvasive catheter no. 1650).[17] All bacterial samples were taken during an infection free period and the detailed results have been published.[18]

COLLECTION OF SAMPLES
All samples were collected between 08.30 and 10.00 hours. Bronchial lavage fluid was collected by a single instillation of 5 ml sterile phosphate buffered saline (PBS) with the bronchoscope in an unwedged position in the anterior segment of the right upper lobe. The fluid was immediately aspirated, collected in a sterile container, and transported to the laboratory where it was frozen at −20°C. Before analysis the fluid was thawed and centrifuged at 10 000 g for 15 minutes. The supernatant was used for analysis as published previously.[19]

ANALYSIS OF INFLAMMATORY MARKERS
Kits for analysis of ECP,[20] MPO,[21] tryptase,[22] and hyaluronan[23] (Pharmacia Diagnostics AB, Uppsala, Sweden) were used according to the instructions of the manufacturers. To ascertain the validity of the assay an analysis of recovery of ECP was performed on three samples and the measured values were within 20% of the expected value (data not shown).

IL-6 was determined with the B9 bioassay previously described.[12 24] Briefly, the B9 cells were added at 5000 cells per well into microtitre plates (Nunc, Roskilde, Denmark) containing dilutions of sample or a standard preparation consisting of recombinant human IL-6. The medium used was Iscoves Modified Dulbeccos Medium (Flow Laboratories, Irvine, UK) supplemented with 50 µM 2-mercaptoethanol, 5% fetal calf serum, and gentamicin (0·1 mg/ml) in a total volume of 0·2 ml. After 68–72 hours of culture ³H-thymidine was added, the incorporated radioactivity was measured and compared with a standard curve. The standard curve was prepared using stock recombinant human IL-6 (8000 units/ml; 1 unit = concentration of IL-6 required for a half maximal thymidine incorporation). The activities of each sample are given as units/ml compared with the standard curve.

IL-8 was analysed using a solid phase double ligand ELISA prototype method. Briefly, the bronchial lavage fluid was incubated in micro-titre wells coated with a mouse monoclonal antibody against IL-8. All samples were diluted in the standard dilution buffer. After washing a goat anti-IL-8 horseradish peroxidase conjugate was added and the plates were incubated. The amount of IL-8 was measured spectrophotometrically after adding tetramethyl-benzidine as enzyme substrate. Recombinant IL-8[25] was used as standard, ranging from 25 ng/l to 1600 ng/l. The intra-assay and inter-assay coefficients of variation were less then 10% and the detection limit was 10 ng/l.

DATA ANALYSIS

Results are expressed as arithmetic mean, median, and standard deviations (SD). The Kruskal-Wallis test was used to analyse the significance of differences in data between the controls, the patients with non-obstructive chronic bronchitis, and the patients with chronic bronchitis and COPD. The Mann-Whitney U test was used for the comparison of the distribution of the inflammatory markers between subgroups of subjects, and Spearman's rank correlation test for possible associations between markers. Multiple regression was per-

formed to analyse the possible interdependence of clinical factors and inflammatory markers. Bonferroni's correction for the risk of mass significance with multiple comparisons between the four subgroups (B(1a), B(1b), B(2a), and B(2b)) was performed.

Results

INFLAMMATORY MARKERS IN CONTROLS AND PATIENTS WITH CHRONIC BRONCHITIS

The patients with chronic bronchitis (group B) had significantly higher mean levels of MPO ($p<0.01$), IL-8 ($p<0.01$), hyaluronan ($p<0.001$), and ECP ($p<0.001$) than the controls (figs 1 and 2). The mean levels of tryptase, IL-6, and albumin did not differ statistically between the patients with chronic bronchitis and the controls.

When albumin values were used as a denominator for each marker the differences in levels of markers between the groups were still present, but the statistical significances were lost (data not shown).

INFLAMMATORY MARKERS IN PATIENTS WITH AND WITHOUT AIRWAYS OBSTRUCTION

The patients with chronic bronchitis and COPD (subgroup B(1b)) did not differ in levels

*Figure 1 Levels of (A) myeloperoxidase (MPO) (µg/l) and (B) IL-8 (µg/l) in bronchial lavage fluid in normal controls (n = 13) and in patients with chronic bronchitis (n = 42). Bold lines represent mean values, dotted lines represent median values. Data are presented on a logarithmic scale. **p<0·01.*
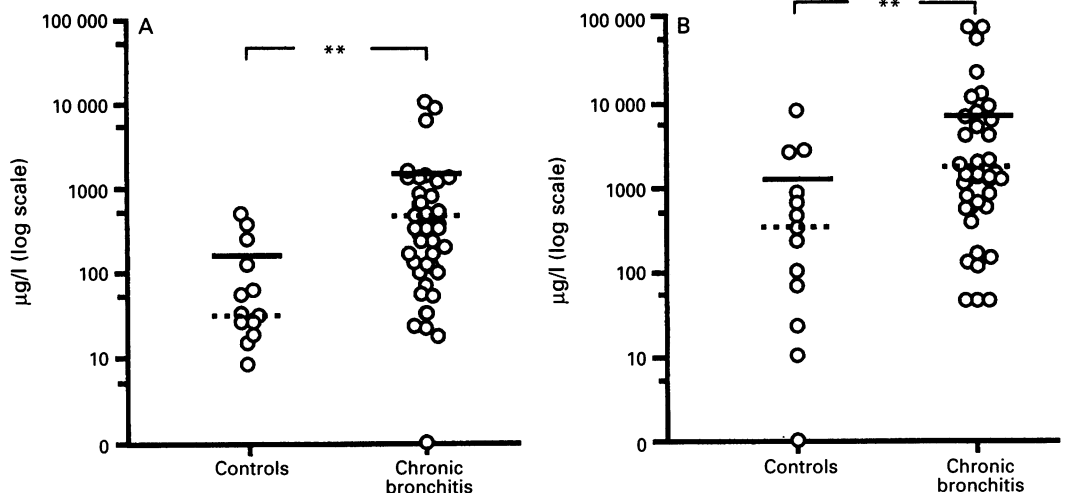

*Figure 2 Levels of (A) hyaluronan (HA) (µg/l) and (B) eosinophil cationic protein (ECP) (µg/l) in bronchial lavage fluid in normal controls (n = 13) and in patients with chronic bronchitis (n = 42). Bold lines represent mean values, dotted lines represent median values. Data are presented on a logarithmic scale. ***p<0·001.*
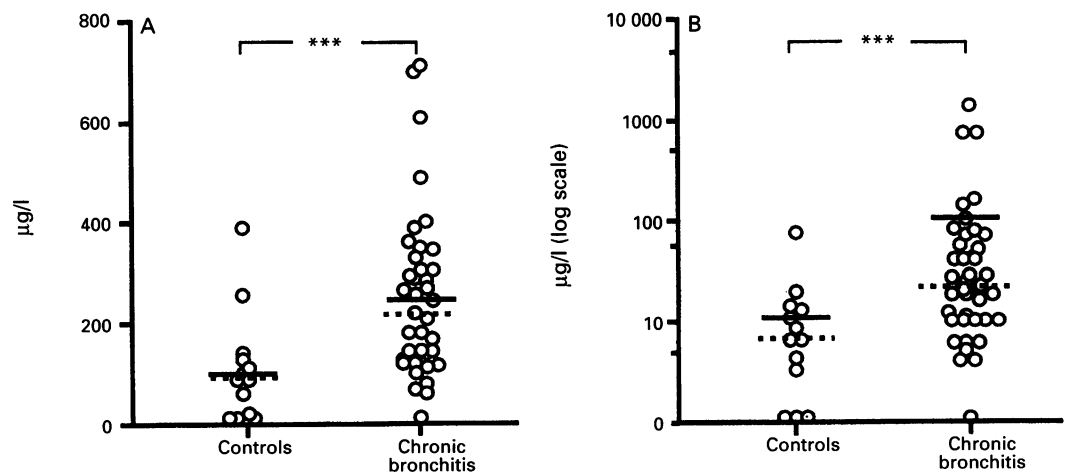
Table 2 Mean (median) levels of inflammatory markers in the controls compared with levels in the four subgroups of patients with chronic bronchitis

Subjects	MPO (µg/l)	IL-8 (µg/l)	HA (µg/l)	ECP (µg/l)	Tryptase (units/l)	Albumin (µg/l)
A Healthy controls	125 (36)	1216 (360)	107 (92)	12 (6)	4 (4)	50 (38)
B(1a) Non-obstructive chronic bronchitis	1028 (280)	5649 (1108)	208 (144)	60 (20)	6·7 (4)	96 (76)
B(1b) Chronic bronchitis with COPD	1263** (400)	10733 (1088)	295** (260)	146** (26)	7·5 (4)	90 (72)
B(2a) Chronic bronchitis without exacerbations	801** (400)	6465 (1120)	190* (157)	99** (24)	5·0 (3)	76 (65)
B(2b) Chronic bronchitis with exacerbations	1506 (220)	9877 (1086)	318** (288)	100** (21)	9·7 (4)	117 (120)

MPO = myeloperoxidase; IL-8 = interleukin-8; HA = hyaluronan; ECP = eosinophil cationic protein.
* p<0·05, ** p<0·01 v controls (Mann-Whitney U test with Bonferroni's correction for multiple comparisons).

of MPO, IL-8, hyaluronan, or ECP compared with the non-obstructive patients (subgroup B(1a)), even though their mean values were higher (table 2). The mean levels of tryptase, IL-6, and albumin were also comparable between the groups.

For the patients with chronic bronchitis and COPD (subgroup B(1b)) the mean levels of MPO, hyaluronan, and ECP were significantly higher than the controls (table 2). The mean level of IL-8 was raised in the patients with chronic bronchitis and COPD, but not significantly so when Bonferroni's correction had been applied. The mean level of tryptase, IL-6, and albumin were comparable between each subgroup of patients with chronic bronchitis and the controls.

INFLAMMATORY MARKERS IN PATIENTS WITH AND WITHOUT INFECTIVE EXACERBATIONS
Patients with chronic bronchitis and recurrent exacerbations (subgroup B(2b)) had a higher mean level of hyaluronan than those without a history of recurrent infections (subgroup B(2a)), but the difference was not statistically significant when Bonferroni's correction had been applied (table 2). No differences were seen in levels of MPO, IL-8, or ECP between bronchitis patients with and without exacerbations, and the mean levels of tryptase, IL-6, and albumin were comparable between the two groups (table 2).

ANALYSIS OF LEVELS OF MARKERS IN RELATION TO INTRABRONCHIAL BACTERIAL GROWTH
Ten patients with chronic bronchitis had significant bacterial growth when the established cutoff level of >10³ colony forming units per ml for the protected specimen brush was used.[17 18] Their mean levels of MPO, IL-8, hyaluronan, and ECP were higher than for the other 32 patients who had low bacterial numbers, but not significantly (data not shown).

Two of the three patients with intrabronchial cultures of Streptococcus pneumoniae were found to have the highest individual levels of MPO, IL-8, and ECP of all subjects in the study (table 3). The levels of markers in the two patients with cultures of Haemophilus influenzae, and in those with significant growth of other bacterial strains, were similar to the levels in the patients without significant intrabronchial bacterial growth (table 3).

The significant differences in mean levels of MPO, IL-8, hyaluronan, and ECP between the patients with chronic bronchitis and COPD compared with the controls were still valid when the high values for the patients with intrabronchial cultures of Streptococcus pneumoniae had been subtracted.

CORRELATIONS BETWEEN PARAMETERS
Highly significant correlations were found between the ECP and MPO levels (rho = 0·74; p<0·001, Spearman's rank test) and the ECP and IL-8 levels (rho = 0·75; p<0·001, Spearman's rank test). The correlation between MPO and IL-8 levels was also highly significant (rho = 0·87; p<0·001, Spearman's rank test) (fig 3). The levels of tryptase, hyaluronan, and albumin showed no correlation with any of the other markers.

None of the clinical paramaeters – age, smoking habits, airway medication including N-acetylcysteine, duration of bronchitis symptoms,

Figure 3 Levels of inflammatory markers in the five patients with intrabronchial bacterial growth of Streptococcus pneumoniae and Haemophilus influenzae. Median levels are given for patients with chronic bronchitis with and without bacterial growth for comparison

Bacteria (cfu/ml)		MPO (µg/l)	IL-8 (µg/l)	HA (µg/l)	ECP (µg/l)	Tryptase (units/l)	Albumin (µg/l)
S pneumoniae	(5 × 10¹)	320	4400	120	56	9	80
S pneumoniae	(2 × 10²)	7360	80000	—	1400	2	148
S pneumoniae	(5 × 10⁴)	10880	80000	704	720	2	152
H influenzae	(3 × 10²)	124	40	60	6	3	10
H influenzae	(3 × 10⁴)	200	1132	360	10	3	38
Other strains (median levels)	(>10³)	240	872	284	10	4	36
Non-significant growth (median levels)		280	1042	176	21	4	71

MPO = myeloperoxidase; IL-8 = interleukin-8; HA = hyaluronan, ECP = eosinophil cationic protein; cfu/ml = colony forming units per ml.

Figure 3 Association between levels of IL-8 (µg/l) and myeloperoxidase (MPO) (µg/l) using Spearman's rank correlation test. A strong positive correlation was found. Data are presented on a logarithmic scale.
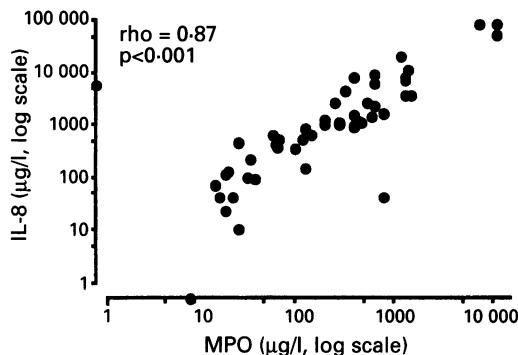
or lung function (FEV_1 percentage predicted normal) – had any correlations with the markers studied.

Discussion

In this study significantly higher activity of neutrophils, fibroblasts, and eosinophils as assessed by levels of MPO, IL-8, hyaluronan, and ECP, respectively, were found in bronchial lavage fluid samples from smokers with chronic bronchitis than from healthy non-smoking controls. However, the levels of tryptase – a mediator known to be related to the activation of mast cells and found in high levels in asthmatics[26] – were similar between the two groups.

Earlier findings in bronchoalveolar lavage fluid from patients with chronic bronchitis have disclosed an increased recovery of macrophages and neutrophils,[27] and the first bronchial fraction of the lavage gives the highest neutrophil numbers.[10] Furthermore, the number of neutrophils in the airways correlated well with airways obstruction (FEV_1) and increased sputum production.[10 27] Our results revealed high levels of MPO, previously shown to be of local origin,[28] in bronchial lavage fluid from patients with chronic bronchitis, suggesting that activated neutrophil granulocytes take part in the airways inflammation in these patients. We also found significantly raised levels of IL-8 in the patients, which is in accordance with a recent study reporting high levels of IL-8 in sputum from patients with chronic bronchitis.[29] IL-8 is a cytokine with chemotactic effects on neutrophil granulocytes[29] produced by alveolar macrophages and respiratory epithelium.[30] The high correlation found between the levels of MPO and IL-8 in the present study supports the hypothesis that a specific attraction and activation of neutrophils is present in the airways inflammatory process in patients with chronic bronchitis.

Our results support those of Linden *et al* who reported increased levels of both ECP and MPO in bronchoalveolar lavage fluid from patients with chronic bronchitis and COPD.[31] The high levels of ECP found in the present study, together with the strong correlations between ECP and MPO, and ECP and IL-8 respectively, support in vitro data that IL-8 not only attracts and activates neutrophils, but also eosinophil granulocytes.[32] Thus, airways inflammation in chronic bronchitis is characterised by a broad activation of mucosal granulocytes including eosinophils.

Free hyaluronan, a marker for activated fibroblasts, was significantly raised in the patients with chronic bronchitis in our study. It was not correlated with airways obstruction, as seen in asthmatic patients,[33] but it was the only mediator to be raised, although not significantly, in patients with a history of recurrent infective exacerbations as opposed to those without.

No specific marker correlated with the development of airways obstruction and COPD. No significant differences were found between the patients with non-obstructive chronic bronchitis and patients with COPD, and no convincing correlations were seen between the levels of markers and FEV_1 values.

Interleukin 6 (IL-6) is a multifunctional cytokine produced by most nucleated cells. It has been found in the urine of patients with asymptomatic bacteriuria[12] and in patients deliberately colonised with *Escherichia coli*.[34] We could, however, find no difference in the levels of IL-6 between the subjects in this study, although several patients had an intrabronchial bacterial colonisation.[18] A possible explanation is the diurnal variation as well as peaking kinetics of IL-6 with intermittent production during long term bacterial colonisation.[12] In our study a single sample was taken from each individual at approximately the same time of day (between 08·30 and 10·00 hours). It is therefore possible that differences in IL-6 secretion could be shown in a longitudinal study.

Two of the three patients with intrabronchial cultures of *Streptococcus pneumoniae* had the highest individual levels of MPO, IL-8, and ECP of all subjects studied (table 3). The levels of markers in the patients with cultures of *Haemophilus influenzae* or α-haemolytic streptococci did not differ from the patients with negative bacterial cultures. This suggests that intrabronchial colonisation of *S pneumoniae* through neutrophil activation may exert a more harmful effect on the mucosa than other bacterial strains commonly found in chronic bronchitis.

To obtain samples which predominantly reflect the status of the bronchial epithelium, small volume lavage (<20 ml) in the large airways is of more value than the traditional large lavage.[13–15] We used a single instillation of 5 ml to minimise the risk of alveolar "contamination". In agreement with Bousquet *et al*[33] we chose to present our data as the concentration of lavaged fluid because (a) the instilled volume was always the same, and (b) no gold standard to compensate for the dilution of the lavaged fluid is valid.[35 36] When we used albumin as the denominator for each marker the differences found between the groups were still present, but not statistically significant. However, previous studies found the use of urea or albumin as denominators of protein ratios for the local concentration of markers in lavaged fluid to be inappropriate.[37 38] The similar levels of tryptase, IL-6, and albumin in the subject groups studied also suggest that our data reflect differences in other markers that are not artefactual.

There are some additional methodological

issues in our study that need to be addressed. Firstly, even if subjects with asthma were excluded, subject atopy was not registered. The similar levels of tryptase in the groups suggest that no large differences in mast cell activation was present, but it is possible that there was some overlap of inflammatory markers in the groups, especially for ECP, which could be due to underlying atopic status in some individuals. In addition, smoking could be a confounding factor in our study since a healthy smoking control group was not included.

Secondly, the assessment of airways inflammation with levels of soluble inflammatory markers in lavage fluid can only be regarded as an indirect evaluation. Our findings have to be substantiated by mucosal bronchial biopsies, and a direct assessment of inflammatory cell activity by immunohistochemical methods.

Thirdly, as cells were not immediately separated from bronchial lavage fluid, it cannot be excluded that intracellular products could have influenced the levels of soluble markers. However, it is unlikely that the differences found between the groups could have been seriously affected since all samples were treated equally, sampled at the same time, and under identical conditions. In addition, our measured differences in ECP and MPO levels are supported by the results reported by Linden *et al.*[31]

In conclusion, mucosal inflammation in chronic bronchitis is characterised by both activated neutrophils as assessed by high levels of MPO, and activated eosinophils as assessed by high levels of ECP. The activation of these granulocytes may be related to the levels of IL-8. Mast cell activation, typical of atopic asthma, could not be demonstrated. Intrabronchial colonisation of *S pneumoniae* was related to high levels of MPO, IL-8, and ECP.

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