

Airways obstruction, chronic expectoration, and rapid decline of FEV₁ in smokers are associated with increased levels of sputum neutrophils

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Abstract

Background – Smoking may cause inflammation of the airways and impairment of lung function. To determine the relationship between the type and degree of airways inflammation and the decline in lung function, leucocytes in the sputum of smokers and ex-smokers were examined.

Methods – Forty six smokers and ex-smokers of median age 64 years (25%;75% percentiles 62;66) with a smoking history of 40.1 (31.7;53) pack years were studied with lung function tests and a questionnaire at the end of a 15 year follow up period. Sputum was induced by inhalation of hypertonic saline and differential leucocyte counts were performed on cytospin preparations.

Results – Adequate sputum samples were obtained in 38 subjects (78%). The ratio of forced expiratory volume in one second (FEV₁) to vital capacity (VC) was 67.1 (60;72)% and the annual decline in FEV₁ was 19.4 (12;30) ml/year. Subjects with airways obstruction (FEV₁/VC <63%) had more neutrophils (77 (50;86)%) than those without airways obstruction (60 (43;73)%). The percentage of neutrophils was also significantly greater (77 (62;85)%) in those with chronic expectoration than in those without expectoration (57 (45;75)%). Increased levels of neutrophils in the sputum were correlated with a rapid decline in FEV₁ over the 15 year follow up period.

Conclusions – Airways obstruction and chronic expectoration, as well as accelerated decline in lung function, are associated with increased numbers of neutrophils in the sputum of smokers and ex-smokers which suggests that neutrophilic inflammation of the airways may be involved in the pathogenesis of chronic obstructive pulmonary disease.

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Keywords: neutrophils, smoking, chronic obstructive pulmonary disease, sputum, lung function.

Patients with chronic obstructive pulmonary disease (COPD) have a considerable clinical and functional heterogeneity with respect to mucus hypersecretion, airways obstruction, and progression of disease. Cigarette smoking

is the most important cause of COPD, but only a few smokers (10–20%) will develop clinically overt disease.¹ The risk for a smoker of developing progressive disease is linked to the rapid decline in forced expiratory volume in one second (FEV₁) with age. The mechanisms for the increased susceptibility in some smokers and the identification early in life of high risk smokers remain unknown.

In earlier studies of COPD emphasis was put on clinical, epidemiological, and functional aspects of the disease. Niewoehner *et al* were among the first to show that smoking produces a respiratory bronchiolitis and alveolitis associated with structural changes in the peripheral airways.² More recent pathological investigations in COPD have found an inflammatory infiltrate with predominantly mononuclear cells in large and small airways,^{3,4} activated T cells and increased macrophages in bronchial mucosa from bronchial biopsy samples,⁵ and intraluminal neutrophilia in bronchoalveolar lavage fluid.^{6–8} Furthermore, upregulation of adhesion molecules, E-selectin on submucosal vessels, and intercellular adhesion molecule-1 (ICAM-1) in bronchial epithelium was detected in “chronic obstructive bronchitis”.⁹ Increased numbers of eosinophils have also been found in the bronchial mucosa, bronchoalveolar lavage fluid and sputum of subjects with chronic bronchitis.^{7,10}

Previous approaches to the study of the pathology of the lung in patients with COPD involved invasive methods. Recently, cell analysis in sputum induced by inhalation of hypertonic saline has been used to investigate airways inflammation.¹¹ The aim of this study was to assess the cellular component of airways inflammation with a non-invasive technique by analysis of induced sputum in a group of smokers and ex-smokers who were clinically and functionally followed up for 15 years. The relationship between smoking habit, chronic expectoration, airways obstruction, and rapid decay of lung function with intraluminal airways inflammation was also investigated.

Methods

SUBJECTS

Forty six former steelworkers, all men, of median age (25%;75% percentiles) 64 (62;66) were examined at the end of a 15 year follow up study. Briefly, in 1978 we studied a group of 104 blue collar workers from a steel plant

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Table 1 Characteristics of the subjects at the start of the study

	Subjects studied over 15 year period (n = 46)	Subjects lost to follow up (n = 58)
Age (years)	49 (47;51)	49 (48;52)
Height (cm)	170 (165;175)	168 (164;173)
Weight (kg)	75 (69;84)	76 (68;81)
VC (% predicted)	91.4 (86.3;97.3)	91.8 (84.5;96.9)
FEV ₁ (% predicted)	86.4 (79.2;92.5)	83.6 (74;95.9)
FEV ₁ /VC (%)	69.8 (64.9;73.2)	69.4 (64.0;74.4)
Pack years	32.6 (21;37.5)	32.7 (22.8;40.3)

Data are expressed as median (25% and 75% percentiles).
VC=vital capacity; FEV₁=forced expiratory volume in one second.

near Brussels (Belgium), all of whom were long term smokers (average 32 pack years). Selection criteria included age 45–55 years and at least 10 years of service in the same company.¹² Workers with bronchial asthma as well as previous occupational exposure to dust were rejected. Before tests of lung function were performed each subject was given the European Coal and Steel Community questionnaire on respiratory symptoms¹³ derived from the Medical Research Council questionnaire. Survivors and those who agreed to be tested again were studied 6, 13, and 15 years later. Forty six of the original 104 subjects, now retired, were studied at the latest examination. Subjects lost to follow up (those who had died, emigrated, or refused to be examined again) did not seem to be different from those seen again 15 years later. Indeed, at the start of the study physical and lung function data, as well as smoking history, were comparable in the two groups (table 1).

Subjects were studied in the morning and were asked not to smoke before the investigation. The administration of the questionnaire was followed by pulmonary function tests and then by induction of sputum. The study conformed to the Declaration of Helsinki and informed written consent was obtained in each subject.

PULMONARY FUNCTION TESTS

Pulmonary function tests at the start of the study, as well as six and 13 years later, included measurements of several lung function tests.^{12,14} At the last session after 15 years only spirometric tests, vital capacity (VC), and forced expiratory volume in one second (FEV₁) were performed. The VC and FEV₁ were taken as the best from at least three satisfactory spirometric tracings. Tests were performed by the same technician using the same water sealed spirometer during the 15 year follow up period. Calibration of the spirometer (with a three litre syringe) and speed of the pneumotachograph (measured with a stopwatch) was done each day on which the subjects were studied. The rate of decline in FEV₁ (ml/year) was calculated for each subject by linear regression of FEV₁ against time. The decline in FEV₁ over time for the group as a whole was calculated as the average value of individual FEV₁ slopes.

INDUCTION OF SPUTUM

Induction of sputum was performed as described by Maestrelli *et al.*¹⁵ Briefly, hypertonic saline was nebulised with an ultrasonic nebuliser (De Vilbiss, Ultra Neb 2000, De Vilbiss Health Care, Somerset, Pennsylvania, USA) for five minute periods up to 20 minutes. The concentration of saline was increased at intervals of 10 minutes from 3% to 4%. Every five minutes subjects were asked to rinse their mouths and throats and then to try to cough sputum into a petri dish. The nebulisation was stopped after 20 minutes or earlier if the size of the sputum sample was estimated to be large enough for its analysis. To detect saline induced bronchospasm, FEV₁ and VC were measured before and 20 minutes after inhalation of saline. If the FEV₁ fell by more than 20% from control values, or if the subjects complained of acute dyspnoea, nebulisation was discontinued and salbutamol, 200 µg by inhalation, was administered.

SPUTUM ANALYSIS

Sputum plugs arising from the lower respiratory tract were selected by visual inspection (to reduce the salivary contamination of the sample) and transferred to a vial. Each sputum sample was incubated with 1 ml dithiothreitol 1% at 37°C for 20 minutes and washed with phosphate buffered saline. The cell pellet was resuspended and the cell suspension was spun in a cytocentrifuge (Shandon Cytospin 2; Shandon, Oakland, California, USA). Two slides were fixed in acetone/methanol (1:1) and stained with May-Grunwald-Giemsa for differential cell counts of leucocytes and squamous epithelial cells. Two further slides were fixed in Carnoy's solution and stained with 0.5% toluidine blue at pH 0.1 for quantification of metachromatic cells (mast cells and basophils). Slides were coded and counted blind by one investigator (PGC). Four hundred cells per slide were counted for differential leucocyte count. For the metachromatic cell count all the available area on each cytopsin preparation which included 4000–24 000 nucleated cells was examined. A sample was considered adequate when the percentage of squamous cells was lower than 50%. To correct for the variability due to salivary contamination, the results of differential leucocyte counts and metachromatic cells were expressed as percentages of nucleated cells excluding squamous cells (average counts of two slides for each patient).

The mean coefficient of variation for at least three repeated measurements was 11% for eosinophils, 7% for neutrophils, 14% for macrophages, and 39% for lymphocytes. The repeatability coefficients of the differential cell counts performed on the two slides of each sample were calculated as twice the standard deviation of the differences between the pairs of measurements¹⁶ which was 9% for eosinophils, 19% for neutrophils, 13% for macrophages, and 4% for lymphocytes.

Table 2 Percentage of leucocytes and squamous epithelial cells in sputum of smokers and ex-smokers

	Smokers (n = 22)	Ex-smokers (n = 16)
Macrophages	27 (14;40)	33 (23;50)
Neutrophils	67 (48;84)	62 (47;76)
Eosinophils	1 (0;4)	1 (1;3)
Lymphocytes	1 (0;2)	0 (0;2)
Metachromatic cells	0.02 (0;0.05)	0.02 (0;0.07)
Squamous cells	12 (0;26)	15 (10;28)

Data are expressed as median (25% and 75% percentiles).

DATA ANALYSIS

Results are presented as median and 25%, 75% percentiles. Since data were not normally distributed a non-parametric test (the Kruskal-Wallis test¹⁷) was used to compare differences between two or more group means in tables 1-3 and fig 2. Correlations between decline in FEV₁ and percentage of sputum leucocytes were examined with the Spearman rank correlation test and significance was accepted at the 5% level.

Results

INDUCTION OF SPUTUM

Adequate induced sputum samples were obtained in 36 of the 46 subjects, two produced sputum spontaneously, and eight were excluded because their sputum samples had more than 50% squamous cells, suggesting excessive salivary contamination. Sputum was therefore analysed from 38 subjects with a median (25%; 75% percentiles) of 64 (62;66) years. Induction of sputum was well tolerated and only in two subjects did the FEV₁ decrease by more than 20% following inhalation of saline.

SYMPTOMS AND LUNG FUNCTION RESULTS

Coughing was reported by 15 subjects, chronic expectoration by 12, wheezing by six, and exertional dyspnoea by 23. For the group as a whole VC was 94.2 (87.4;94.2)%, FEV₁ 85.1 (77.6;98.4)%, and FEV₁/VC 95.0 (85.1; 102.3)% of predicted values.¹⁸ The smoking history of the 38 subjects was 40.3 (29.5;53.0) pack years. Sixteen had given up smoking at various intervals following the start of the study and 22 continued to smoke. The FEV₁/VC ratio (% of predicted) was 86.5 (80.3;99.4)% in the former group and 102.1 (95.1;108.0)% in the latter (p<0.05).

Table 3 Percentage of leucocytes in sputum according to the presence (or absence) of chronic expectoration (E) and/or airways obstruction (A)

Group	Macrophages	Neutrophils	Eosinophils
A+/E+ (n=4)	12 (9;16)	86 (83;90)	0.5 (0;2)
A-/E+ (n=8)	28 (22;38)	69 (55;77)	2 (0.5;3)
A+/E- (n=13)	24 (14;39)	66 (48;83)	1 (0;14)
A-/E- (n=13)	45 (28;53)	54 (43;68)	1 (1;5)

Data are expressed as median (25% and 75% percentiles).
*p<0.05; **p<0.01.

SPUTUM CELLS

The percentages of leucocytes and squamous cells in the sputum of smokers and ex-smokers are presented in table 2. There were no significant differences in the percentage of neutrophils or of other cells between the two groups. Similarly, smoking history, expressed as the number of pack years smoked, showed no significant relationship with the sputum cells.

There was a significantly higher percentage of neutrophils (p<0.05) in those who reported chronic expectoration (77 (62;85)%) than in those who did not (57.0 (45;75)%). Similarly, the percentage of neutrophils was significantly higher (p<0.05) in those with airways obstruction (77 (50;86)%) than in those without (60 (43;73)%). Subjects with airways obstruction and chronic expectoration had more neutrophils (p<0.05) than those without airways obstruction (with or without expectoration), and also more neutrophils than subjects with airways obstruction but without chronic expectoration (table 3). Airways obstruction was defined as an FEV₁/VC ratio of less than 63.3% - that is, the cutoff limit of FEV₁/VC ratio at the start of the study (66.6%) less the annual decline of this ratio estimated from ref 18.

The percentage of eosinophils was significantly increased in the seven subjects with severe airways obstruction (FEV₁/VC <55%) compared with all others combined as one group (3 (0.3;20.3)% versus 1 (0;3)% respectively). Since the percentages of metachromatic cells and lymphocytes were very low, their values are not reported.

DECLINE IN FEV₁ AND RELATIONSHIP WITH SPUTUM CELLS

The average decline in FEV₁ for the group as a whole over 15 years was 19.4 (12;30) ml/year. The frequency distribution of the loss in FEV₁ is presented in fig 1. In most subjects there was a moderate decrease in FEV₁, in a few there was little or no change, while in some the fall in FEV₁ exceeded 40 ml/year. The mean FEV₁/VC ratio in the latter subjects was as low as 40%. Loss of FEV₁ was higher in smokers (25.2 (17;32) ml/year) than in ex-smokers (17.1

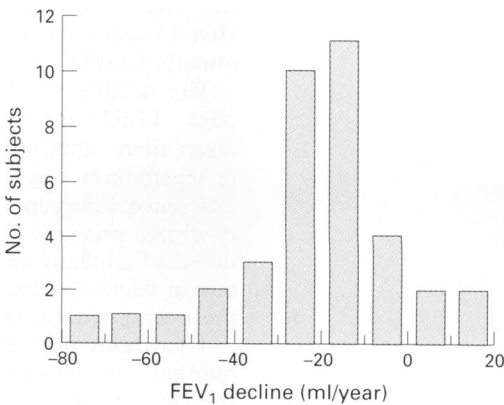


Figure 1 Distribution of rates of annual decline in forced expiratory volume in one second (FEV₁) in the 38 subjects.

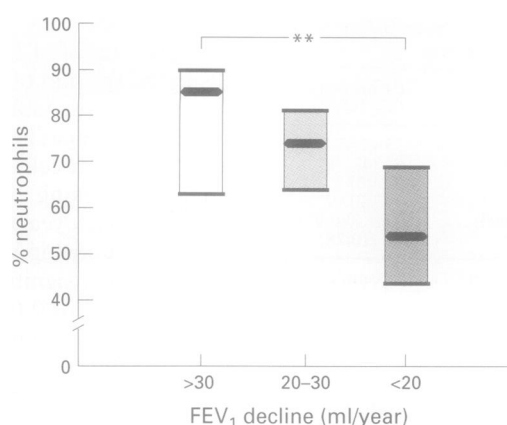


Figure 2 Percentage of neutrophils in sputum (median, 25% and 75% percentiles) in subjects with a decline in forced expiratory volume in one second (FEV₁) of <20 ml/year, 20–30 ml/year, and >30 ml/year. ** $p < 0.01$.

(8;25) ml/year) but this difference was not significant, probably due to the dispersion of data.

The relationship between the rate of decline in FEV₁ and the percentage of leucocytes in the sputum is presented in fig 2. The decline in FEV₁ was correlated with the percentage of neutrophils ($r = 0.4$, $p < 0.01$) and inversely related to the percentage of macrophages ($r = 0.5$, $p < 0.01$).

Discussion

On four occasions over 15 years a group of smokers and ex-smokers has been studied using lung function tests and a questionnaire on respiratory symptoms. Cell counts in induced sputum were used to characterise the subjects at the end of the study. We have found that chronic expectoration, airways obstruction, and rapid decline in lung function were associated with increased numbers of neutrophils in the sputum.

The average decline in FEV₁ over time for the group as a whole was 19.4 (12;30) ml/year. In most subjects there was a moderate decline in FEV₁, but in a few there was little or no change and in some others the decline in FEV₁ was rapid, reaching FEV₁/VC ratios as low as 40% (fig 1). A few subjects who might be considered the high risk group – active industrial workers in their fifties at the start of the study – thus reached a functional profile after 15 years similar to that observed in symptomatic COPD.

The decline in FEV₁ in smokers averaged 25.2 (17;32) ml/year (comparable to^{19,20} or lower than²¹ that found by others) while that of ex-smokers was 17.1 (8;25) ml/year. The difference between smokers and ex-smokers confirms previous observations^{22,23} that cessation of smoking results in a decrease in the rate of decline in FEV₁ compared with that in those who continue to smoke.

There were no significant differences in the numbers of neutrophils in the sputum of smokers and ex-smokers and the cell profile of both groups is consistent with a chronic neutrophilic inflammation of the airways. It is therefore unlikely that the influx of neutrophils

into the airways is an acute response to smoking. These results are in agreement with previous findings that have shown that airways inflammation induced by tobacco smoke may persist independently from smoking.^{24–27} It appears therefore that smoking is responsible for triggering inflammation of the airways which may, however, be self-perpetuating even after cessation of smoking.

Chronic mucus hypersecretion and airways obstruction are the two major characteristics of COPD. In a longitudinal study of middle aged male workers Fletcher *et al*¹ found that mucus hypersecretion and airways obstruction might be associated, but they are not causally related. In a random sample of the general population one can therefore find subjects with airways obstruction or chronic mucus production only, subjects with both, and subjects without either of these defects. In the present study the highest percentage of neutrophils was found in subjects with both airways obstruction and chronic expectoration, and other groups had lower percentages of neutrophils (table 3). Despite the small number of subjects in the two subgroups, the differences were significant using non parametric tests. Thompson *et al*⁶ have also found that smokers with more neutrophils in the bronchoalveolar fluid had significantly more sputum production and a lower FEV₁ and FEV₁/VC ratio than did subjects with fewer bronchial neutrophils.

Increased numbers of neutrophils were detected in the sputum of subjects with a rapid decline in FEV₁ over the 15 year follow up period. Other studies have found high percentages of intraluminal neutrophils in patients with chronic bronchitis^{8,28,29} and have reported an association of airway neutrophilia with airways obstruction in both bronchoalveolar lavage fluid^{6,8,29} and bronchial biopsy specimens.² However, this is the first report of an association between an accelerated decline in FEV₁ and excess sputum neutrophilia (fig 2) – for example, subjects with >70% neutrophils in the sputum had a decline in FEV₁ (27 ml/year) that was twice that observed in subjects with <70% neutrophils (12 ml/year).

In addition to neutrophils, eosinophils also characterise bronchial inflammation in patients with chronic bronchitis.^{7,10} In our subjects increased numbers of eosinophils in the sputum were detected in those with more severe airways obstruction, suggesting that eosinophils may be related to more severe or exacerbated disease. Although asthmatic subjects were discarded at the beginning of the study, we cannot be sure that some subjects did not develop asthma later in the follow up period.

Mast cells, as well as lymphocytes, are found mainly in the submucosa of healthy, asthmatic, and bronchitic subjects.^{5,30,31} However, their level in the sputum of our subjects was very low, suggesting that mast cells and lymphocytes do not migrate toward the lumen of the airways to the same extent as do granulocytes and macrophages.

Our results confirm and support previous findings¹¹ that hypertonic saline-induced sputum can be used in the assessment of airways

inflammation. Sputum was induced by inhalation of saline in 36 of the 46 subjects examined (78%) which compares favourably with the 76% adequate samples reported by Pin *et al*¹¹ in 17 asthmatic patients and 17 healthy subjects. Fahy *et al*³² have recently reported that analysis of induced sputum in healthy and asthmatic subjects reveals information qualitatively similar to that obtained by analysis of bronchial washing and bronchoalveolar lavage, yielding samples that are more concentrated and richer in airway secretions than those obtained by bronchoscopy. Since it is non-invasive this method may provide a useful tool for the examination of inflammatory processes in the airways in large surveys or in studies requiring repeated sampling of the airways.

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