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Eosinophils in induced sputum from asymptomatic smokers with normal lung function

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Abstract Cigarette smoking is the dominant risk factor for chronic obstructive pulmonary disease (COPD). However, only 10–15% of smokers develop the disease and early changes within the airways are poorly defined. We aimed to compare cell profiles in induced sputum (IS) from asymptomatic smokers to that from healthy subjects, and to ascertain whether or not inflammatory cells in IS are related to lung function and smoking habit. We recruited 34 heavy, non-allergic asymptomatic smokers with normal lung function and 15 healthy volunteers, who performed lung function tests and IS by hypertonic saline (3%) solution. In smokers, significant correlation between pack-years and FEF_{25-75} ($r_s = -0{\cdot}43$, $P < 0{\cdot}02$) was found. In IS, smokers had higher counts of macrophages ($P < 0{\cdot}01$) and eosinophils ($P < 0{\cdot}02$), when compared to those of healthy subjects. Additionally, eosinophils were found in IS of 14 out of 34 smokers, with eosinophils had a higher pack-years (31 ± 25 *vs.* 13 ± 10, $P = 0{\cdot}02$) and lower $FEF_{25-75\%}$ value ($78\% \pm 34$ *vs.* $100\% \pm 23$, $P < 0{\cdot}04$), when compared to smokers without eosinophils. Additionally, on the basis of regression equations by stepwise multiple regression analysis, eosinophils were predicted by pack-years ($r^2 = 0{\cdot}41$). Our results showed that asymptomatic smokers have evidence of inflammatory cells in IS samples. In addition, we found that the degree of eosinophilic inflammation is related to early changes of lung function and can be predicted by smoking habit.

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Keywords induced sputum; eosinophils; smoking habit; lung function.

INTRODUCTION

Cigarette smoking is the dominant risk factor for chronic obstructive pulmonary disease (COPD) (1,2). However, only 10–15% of smokers develop the disease (1) and early changes within the airways are poorly defined. A decrease in forced expiratory volume in 1 sec (FEV_1) and forced mid-expiratory flow (FEF_{25-75}) have been recognized as early lung function changes in smokers without COPD (3–6). In smokers with chronic bronchitis the percentage of neutrophils in bronchoalveolar lavage (BAL) is higher than in asymptomatic smokers or the normal subjects (7). The percentage of neutrophils in BAL correlated with the number of pack-years, sputum production and airway obstruction (7). In addition, smokers have a higher number of alveolar macrophages from BAL fluid with numerous morphological and functional changes compared to non-smokers (8). BAL fluid from COPD patients is characterized by an increase in activated lymphocytes (9), neutrophils (10), and macrophages (9), as well as a mild degree of eosinophilia (11).

Induced sputum (IS), an alternative method to assess airway inflammation (12) has long been performed in smoking individuals. The percentage of neutrophils in IS from smokers with airway obstruction was higher than that found in smokers without airway obstruction (13). In addition, the percentage of neutrophils in IS correlated with the decline in FEV_1, assessed over a 15-year follow-up period (13). The phenotype of sputum macrophages was altered by chronic smoking (14), and the expression of integrins on sputum polymorphonuclear leukocytes was increased (15). It has been suggested that the over-expression of integrins could represent a marker for smokers who are more susceptible to develop COPD (15).

The aim of this study was to compare cell profiles in IS from asymptomatic smokers to IS from healthy subjects and to ascertain whether or not inflammatory cells in IS are related to lung function and smoking habit.

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METHODS

Subjects

We enrolled 34 (17 female, age range 19–71 years) heavy, non-allergic smokers (2·5–80 pack-years) from our outpatient clinic. Subjects had no history of chronic hypersecretion, asthma, chronic bronchitis or any allergic disease. The atopic status was also assessed by blood eosinophil count and by skin prick tests. Blood samples were obtained in all subjects and leukocyte counts and standard differential eosinophil counts were estimated by a Coulter counter (Sysmex SE-9500, Toa, Japan). We selected only subjects with normal percentages of blood leukocytes and eosinophils. Skin prick tests were performed by using a standard battery of eight common airborne allergen extracts.

Moreover, all subjects did not have any bronchial or respiratory tract infections during the month preceding the study. Baseline lung functional tests were within the normal range (FEV_1 value $>85\%$ of predicted value and $FEV_1/VC>80\%$; changes in FEV_1 20 min after the inhalation of 200 μg salbutamol $<12\%$ as percentage of predicted value).

As a control group, we included 15 healthy lifetime non-smoking volunteers recruited from the hospital staff (10 female, age range 21–70 years) who had experienced no acute respiratory illness within the 4 weeks prior to the study. All subjects denied personal or family histories of allergy or respiratory disease. Each subject gave informed consent to study protocol.

Study protocol

Subjects were studied in the morning (between 08·00 and 10·00 hours) and were asked not to smoke during the preceding 12 h. Lung function measurements and induced sputum were performed on the same day.

Lung function tests included measurement of vital capacity (VC), FEV_1 and FEF_{25-75} (Vmax 22, Sensor Medics, Yorba Linda, U.S.A.).The normal reference values were those proposed by The European Respiratory Society (16). Sputum induction and processing were carried out according to Fahy's method (12), with slight modifications (17). All subjects inhaled 3% hypertonic saline solution four times for 5 min using an ultrasonic nebulizer (Heyer Orion 1, BAD EMS; mean volume output: 2·40 ml min^{-1}). Throughout the procedure, subjects were encouraged to cough and to expectorate into a plastic container. Three flow volume curves were performed before and after each inhalation and the best FEV_1 was recorded. Induction of sputum was stopped if FEV_1 value fell by at least 15% from baseline or if troublesome symptoms occurred.

Sputum sample volume was measured and an equal volume of dithiotreitol 0·1% was added and incubated at 37°C for 30 min. Ten microlitres of the homogenized sample were used to determine total cell counts and results were expressed as number of cells $\times 10^5$ ml^{-1}. The remaining sputum was washed with phosphate-buffered saline and centrifuged at 2000 rpm for a 5-min period. The supernatant was aspirated, and cell pellets were re-suspended in saline (2000 rpm for 10 min), cytocentrifuged at 600 rpm for 10 min and stained with May–Grünwald–Giemsa. The percentage of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells were counted on at least 400 cells, excluding squamous cells. The IS samples were considered adequate if salivary contamination were not excessive (more than 80% of squamous epithelial cells).

Statistical analysis

The percentage of cells was reported as mean $\pm$ SD. The goodness of fit to the normal distribution was assessed by Kolmogorov–Smirnov test. Smokers were grouped in two subgroups, according to the presence of the eosinophils, defined by the count ≥ 1 on at least 400 cells from IS. Differences in numerical data between groups were analysed by unpaired t-test or by Mann–Whitney U-test, when appropriated. Differences in qualitative data were analysed by Fisher exact test. Relationships were estimated by Spearman rank correlation coefficient (r_s). Stepwise multiple regression analysis was used to determine the best predictor variables among age, packs/year, FEV_1, VC, FEV_1/VC, and FEF_{25-75} for the dependent variable eosinophils. Percentage of total variance in the dependent variable, accounted for by the predictors variables is expressed as the adjusted square of the multiple correlation coefficient (r^2). To avoid co-linearity, variables affected by airflow obstruction (for example FEV_1, FEV_1/VC, and FEF_{25-75}) were entered into the analysis separately. A P-value of less than 0·05 was considered significant.

RESULTS

Characteristics of asymptomatic smokers and healthy subjects are shown in Table 1. No differences were found between smokers and healthy subjects in age, gender, and in FEV_1, VC, FEV_1/VC, but not in FEF_{25-75} values.

In smokers, the mean count of leukocytes ($n \times mm^3$) and the mean percentage eosinophils from blood sample were 6170 ± 1990 and $1.5\% \pm 1$, respectively. In these subjects, a significant correlation between pack-years and baseline FEF_{25-75} values ($r_s=-0·43$, $P<0·02$) was also found.

The procedure for sputum induction was well tolerated and adequate sputum samples were obtained in all subjects. The total and differential cell counts of IS between smokers and healthy subjects are presented in

Table 1. Characteristics of the smokers and healthy subjects

	Smokers (n=34)	Healthy subjects (n=15)
Gender (M/F)	17/17	5/10
Age (yrs)	40±14	38±15
VC (% pred)	113±18	108±12
FEV_1 (% pred)	110±20	112±13
FEF_{25-75} (%pred)	91±30*	109±18
FEV_1/VC (%)	81±10	85±10
Pack-years (n)	21±20	—

Values are expressed as means ± SD.
*$P<0.04$, unpaired t-test.

Table 2. Smokers had higher counts of macrophages ($P<0.01$) and eosinophils ($P<0.02$), when compared to those of healthy subjects. Additionally, in smokers, no correlation was found between blood and IS eosinophils.

Eosinophils were found in IS of 14 out of 34 smokers. When smokers were grouped in two subgroups, according to the presence of the eosinophils, smokers with eosinophils had a higher pack-years (31±25 *vs.* 13±10, $P<0.02$) and lower $FEF_{25-75\%}$ value (78%±34 *vs.* 100%±23, P=0·04), when compared to smokers without eosinophils (Fig. 1). Moreover, in the former subgroup FEV_1 values were numerically but not statistically lower than those of the latter subgroup (102%±22 *vs.* 115%±18) (Table 3).

The regression equation generated by stepwise multiple regression analysis for eosinophils, as dependent variable, included pack-years, as the main predictor variable: eosinophils (%)=0·13+0.12 (pack-years); (r^2=0·41).

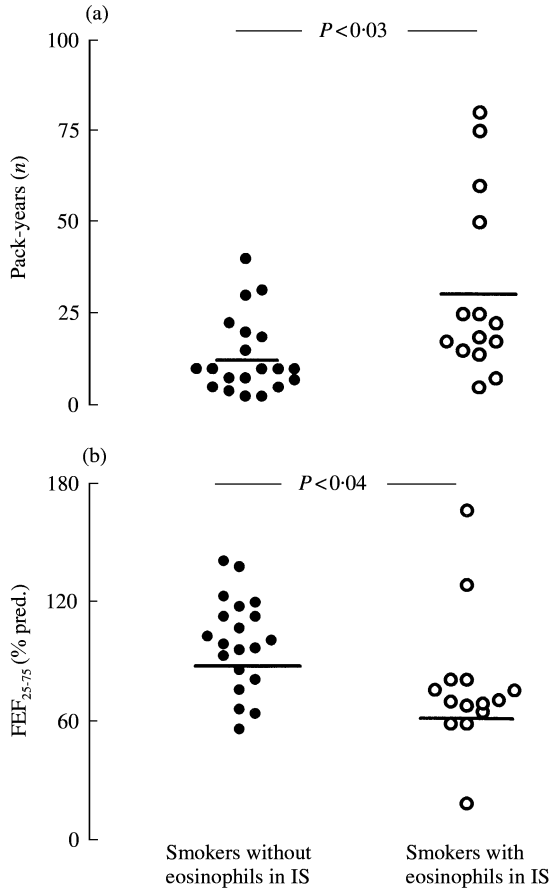

Fig. 1. (a) Individual and mean values of number of pack-years in 20 smokers without eosinophils in IS and in 14 smokers with eosinophils in IS ($P<0.02$, Mann–Whitney U-test). (b) Individual and mean values of FEF_{25-75} value in 20 smokers without eosinophils in IS and in 14 smokers with eosinophils in IS ($P<0.04$, unpaired t-test).

DISCUSSION

In this study, we showed that eosinophils can be present in IS from asymptomatic smokers with normal lung function. We also found that the presence of eosinophils is strictly linked to early changes in lung function, assessed by $FEF_{25-75\%}$, and can be predicted by smoking habit.

Table 2. Total cell and differential cell count in induced sputum from 34 smokers with normal lung function tests and 15 healthy subjects

	Smokers	Healthy subjects
Total cells, $n \times 10^5$ ml^{-1}	5·2 (1·0–25·6)	3·1 (0·9–8·2)
Squamous cells, $n \times 10^3$ ml^{-1}	170 (0·3–740)	210 (50–600)
Macrophages, $n \times 10^3$ ml^{-1}	250 (3–1750)*	80 (3–170)
Neutrophils, $n \times 10^3$ ml^{-1}	90 (0·1–870)	20 (0·1–110)
Lymphocytes, $n \times 10^3$ ml^{-1}	0·4 (0–50)	0·4 (0–10)
Eosinophils, $n \times 10^3$ ml^{-1}	0·2 (0–20)*	0·02 (0–0·2)

Values are expressed as mean (range).
*$P<0.05$, Mann–Whitney U-test.

Table 3. Characteristics of the smokers with eosinophils in the IS and the smokers without eosinophils in IS

	Smokers with eosinophils (n=14)	Smokers without eosinophils (n=20)
Gender (M/F)	10/7	7/13
Age (yrs)	44±16	37±12
VC (% pred)	111±18	114±18
FEV_1 (% pred)	102±22	115±18
FEF_{25-75} (%pred)	78±34*	100±23
Pack-years (n)	31±25†	13±10

Values are expressed as means ± SD.
*$P<0{\cdot}05$, unpaired t-test; †$P<0{\cdot}05$, Mann–Whitney U-test.

We found that the eosinophil count in IS from asymptomatic smokers was significantly higher than that from age and gender matched controls, even if it does not differ when compared to that of large numbers of normal subjects (18,19). However, the applicability of these reference values to our study is limited both by different sputum induction protocol, particularly in terms of different hypertonic saline concentration and duration of inhalation, by different method of sputum processing, and by different age ranges. The presence of eosinophils in IS from smokers is not surprising. Keating and Barnes (20) have detected an increased concentration of eosinophil cationic protein (ECP) and of eosinophil peroxidase (EPO) in IS from smokers with airflow I imitation. These findings suggest that eosinophils in the airway of airflow limited patients are actively degranulating. In addition, it has been demonstrated that the number of eosinophils in sputum and in bronchial biopsies tends to increase during an exacerbation in COPD patients (11). A possible role for eosinophils in the airway inflammation induced by smoking is also supported by animal studies. Repeated exposure to cigarette smoke may induce prolonged airway inflammation and airway hyper-responsiveness in guinea-pigs. Compared to control animals, eosinophils were observed frequently in the mucosa of the main bronchi of cigarette smoke-exposed guinea-pigs (21). Furthermore, in smokers with severe COPD and airway obstruction, a significant improvement in FEV_1 after oral prednisone is evident only in those patients with sputum eosinophilia ($>3\%$) and treatment is associated with a significant decrease in sputum eosinophilia and ECP (22). Moreover, eosinophils in sputum were inversely related to FEV_1, FEV_1/VC, and bronchial hyper-responsiveness, and directly related to clinical score (23). Also, eosinophils in the blood might be a risk factor for chronic air-flow limitation among adult non-smokers (24). However, there is still some debate concerning the number and possible significance of eosinophils in the airway of patients with COPD. In fact, although airway eosinophilia has been found in COPD during exacerbation (11), an increase in airway eosinophils in patients with stable conditions has been found in some studies (20,25), but not in others (26). This could be due, at least in part, to the different criteria adopted for patient inclusion.

In this study, smokers had a significantly higher percentage of macrophages in their sputum samples compared to those of controls. This finding is consistent to previous reports on induced sputum from smokers (14,20) and could be likely due to a decreased clearance of macrophages from the airways as well as an increased recruitment of blood monocytes to the alveoli. In contrast with previously reported data, we found no association between neutrophils in sputum, airway patency degree and smoking history. Previous observations have found that an increased number of neutrophils in the sputum was associated with the degree of airway obstruction and with a more rapid decline in lung function in smokers and ex-smokers (13). Moreover, neutrophils percentages in the smokers with COPD were significantly correlated with the degree of airflow limitation as expressed as percent of predicted FEV_1 (23). In COPD patients and in asymptomatic smokers, neutrophils were related to FEV_1 and FEV_1/VC ratio (27). Moreover, in the same study, significant correlations were observed between neutrophils and eosinophils in the induced sputum, suggesting an interplay between these cells in chronic airway inflammation due to smoking habit (28). Additionally, in smokers with COPD, the sputum concentration of IL-8, chemokine involved in the migration and activation of neutrophils and eosinophils, was significantly correlated with the concentration of ECP and negatively correlated with FEV_1 and FEV_1/VC (29). However, the concentration of IL-8 did not differ significantly between the current smokers and ex-smokers. These findings suggest that an abstention from smoking does not alter the activation of the airway inflammation cycle (30). In fact, no significant differences in lung function, leukocyte differential, or metachromatic cell counts were observed between current and ex-smokers or between subjects with and without chronic expectoration. In addition, smokers with airway obstruction tended to have more neutrophils and fewer macrophages in sputum compared with those with normal FEV_1/VC ratio (15). However, this ratio did not reflect the narrowing of the small airways. Small airway disease was considered not only as early disease, but also a first stage in a long process leading eventually to chronic airflow obstruction. Since early disease cannot be detected by measuring FEV_1 because middle-aged smokers are at no evident risk of functional deterioration if their FEV_1/VC ratio is normal (31). Thus, in our smokers, we considered FEF_{25-75} and saw that

there was a strict link between eosinophils, pack-years and FEF_{25-75}. Although neutrophils in BAL fluid and IS correlated positively with pack-years (23) we did not observe any other correlations with inflammatory cells in IS. A significant inverse correlation between the FEV_1 and pack-years and significant correlation between the FEV_1 and neutrophils in BAL fluid (31) and in IS (32) were also observed. The differences between our data and those of other investigators is probably based on the selection of the smokers, since previously observations considered smokers with COPD. Our population also included asymptomatic smokers without airflow limitation who might be considered as intermediate between COPD and healthy status.

In conclusion, our results showed that in IS samples from smokers without chronic cough and expectoration, there is evidence for the presence of inflammatory cells (macrophages and eosinophils). In addition, we found that the percentage of IS eosinophlis can be predicted by the smoking habit. Therefore, our study suggests that IS can be a potential tool for defining early cytological changes in asymptomatic smokers.

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