

The cellular composition of induced sputum in chronic obstructive pulmonary disease

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ABSTRACT: Asthma and chronic obstructive pulmonary disease are characterized by airway inflammation, which can be assessed by bronchoscopic techniques as well as by the analysis of induced sputum.

A method to induce sputum with inhaled hypertonic saline was adapted for use in 21 chronic obstructive pulmonary disease (COPD) patients (mean baseline forced expiratory volume in one second (FEV₁) 1.60 L, or 54% predicted) and in 16 healthy volunteers. The success rate and safety of the method, were investigated along with the reproducibility of cell counts and differences in cell counts between both groups.

All subjects produced adequate samples and the procedure did not alter spirometric values. A marked sputum neutrophilia was noted in patients with COPD (74.9±4.7%), whereas mainly macrophages were seen in healthy volunteers (74.0±4.0%). Reliability of the cell counts was high, both within investigators (r=0.99 neutrophils, r=0.99 macrophages) and between investigators (r=0.95 neutrophils, r=0.77 macrophages). In patients with COPD, an inverse correlation was noted between percentage of neutrophils and FEV₁ (r_s= -0.48, p<0.05). Immunostaining revealed a large proportion of activated macrophages in both groups.

It was concluded that induction of sputum is a safe and reproducible method to study the composition of airway secretions in patients with chronic obstructive pulmonary disease.

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Inflammation plays a key role in the pathophysiology of asthma [1] but whether it is also central to the pathophysiology of other forms of chronic obstructive airway disorders, such as chronic obstructive pulmonary disease (COPD) and chronic bronchitis remains to be fully investigated. In biopsies of patients with asthma primarily eosinophils, monocytes and T-lymphocytes [2, 3] are seen, but a predominant infiltration of mononuclear inflammatory cells with few polymorphonuclear cells was seen in surgically resected specimens of patients with chronic bronchitis [4]. Moreover, in lobar bronchial biopsies of subjects with chronic bronchitis, an increased number of leukocytes, both in the epithelium and in the lamina propria, consisting predominantly of macrophages and activated T-cells was found [5]. In order to overcome the difficulties associated with bronchoalveolar lavage and the sampling of bronchial biopsies *via* the bronchoscope, analysis of spontaneously produced or induced sputum has recently been suggested for repeated evaluation of airway inflammation in patients with asthma [6–8], chronic bronchitis [7–9] and COPD [9]. The aim of the present study was to assess the success rate, reproducibility and safety of a method for sputum induction in patients with severe COPD and to compare the differential cell counts and immunocytochemical analysis with sputum obtained from healthy volunteers (HV).

Material and methods

Subjects

In a prospective study 21 patients with a poorly reversible airflow obstruction were analysed (table 1). Their diagnosis was based on consensus criteria [10]. None of the patients had a respiratory tract infection within the 4 weeks preceding the procedure. The patients pre-bronchodilator forced expiratory volume in one second (FEV₁) was 1.6±0.1 L (mean±SEM) or 54% predicted, with an increase in FEV₁ <10% pred after 400 µg salbutamol. All patients were exsmokers (stopped at least 1 yr before study entry). They had smoked for a mean 37.0±2.7 pack-yrs. The healthy controls (sex, but not age-matched) were selected from the hospital employees and were nonatopic nonsmokers with a normal pulmonary function. All subjects of both groups had negative skin tests for a panel of allergens. They gave informed consent and the study was approved by the ethics committee of the University Hospital.

Study design

Subjects visited the laboratory three times. On the first visit subject characteristics were documented and lung

Table 1. – Individual data of the chronic obstructive pulmonary disease subjects

Subject No.	Sex	Age yrs	FEV ₁ L	FEV ₁ % pred	FEV ₁ /VC %	Smoking pack-yrs	DL _{CO} /VA % pred	Medication
1	M	76	1.13	53	53	60	52	IS, B
2	M	53	1.36	51	51	25	67	IS, B
3	M	68	2.07	72	67	40	97	IS, B
4	M	68	1.24	43	31	47	50	IS
5	M	45	1.77	55	45	20	64	IS, B
6	M	57	2.81	80	59	40	62	IS, B
7	M	64	2.04	65	58	30	56	IS
8	M	67	2.03	71	53	40	68	IS, B
9	F	60	1.84	68	62	40	58	IS
10	M	56	1.44	36	54	60	91	IS
11	M	52	1.31	39	56	55	79	
12	M	60	1.28	43	50	40	42	IS
13	M	58	0.71	23	22	35	33	IS, B
14	M	52	1.50	43	43	35	50	IS, B
15	F	56	1.65	59	53	20	56	IS
16	M	67	1.73	62	67	20	75	IS
17	M	69	1.53	50	40	35	51	IS
18	M	63	2.13	64	54	30	55	IS
19	M	63	1.66	49	62	20	71	IS
20	M	63	1.64	55	56	30	73	IS, B
21	M	69	1.62	53	64	50	52	IS, B
Mean		61	1.6	54	53	37	62	
SEM		1.6	0.1	3	2.5	2.7	3.4	

FEV₁: forced expiratory volume in one second; VC: vital capacity; DL_{CO}/VA: diffusion capacity for carbon monoxide per litre/lung (alveolar) volume; IS: inhaled steroids ($\leq 1,000 \mu\text{g}\cdot\text{day}^{-1}$); B: inhaled β_2 -adrenoceptor agonists *p.r.n.*; M: male; F: female.

function measurements were performed according to a standardized protocol. Subsequently, subjects underwent sputum induction, at the same time of the day, on 2 days (with an interval of 10 days). Spirometric values were recorded before and after induction by means of a water-sealed spirometer (Expirograph®; Godard, Beethoven, The Netherlands).

Sputum induction

After the inhalation of salbutamol ($2 \times 200 \mu\text{g}$), subjects were asked to inhale sterile, pyrogen-free, hypertonic saline in increasing concentrations (3.0, 4.0 and 5.0%, respectively), for a duration of 10 min each. The hypertonic saline was nebulized *via* an ultrasonic nebulizer (Ultra-neb; Devilbiss Health Care Inc, Somerset, PA, USA). Subjects were encouraged to cough throughout the procedure. Most patients were able to expectorate an adequate sample (at least 5 mL) within the first 10 min.

Sputum processing

Sputum analysis was performed as previously described [6] and validated [11]. In order to reduce salivary contamination, plugs were selected and transferred into an Eppendorf tube. Freshly prepared 10% solution of dithiothreitol (1mL) (DTT; Sputalysin, Boehringer-Calbiochem Corp, San Diego, CA, USA) was added. The tube was vortex mixed and the sputum was incubated for 5 min at room temperature, filtered through 52 μm nylon gauze (BNSH Thompson, Scarborough, Ontario, Canada) to remove debris and mucus, and subsequently centrifuged at $450 \times g$ for 10 min. The cell pellet was resuspended in

phosphate-buffered saline (PBS) in a volume equal to the sputum plus DTT solution volume.

Total cell counting was carried out in a haemocytometer and the cell concentration was adjusted to 1.0×10^6 cells·mL⁻¹. Cytospins were prepared by adding 75 μL cell suspension into Shandon II cytocentrifuge cups (Shandon Southern Instruments, Sewickley, PA, USA) and spun for 6 min at 450 rpm. Two slides were stained with Wright–Giemsa for an overall differential cell count of leukocytes, bronchial epithelial cells and squamous cells. Slides were coded and counted blind by two investigators. None of the cytospins contained >20% squamous epithelial cells. For cell differentiation, 400 nucleated cells per slide were counted and expressed as percentage of intact round nucleated cells, excluding the squamous epithelial cells.

Immunocytochemical analysis

Cytospins obtained from eight healthy persons and from eight subjects with COPD were selected at random, based solely on a homogeneous dispersion on the Wright–Giemsa stained slides, for immunostaining. Cell surface markers were identified by immunocytochemical staining with antibodies against CD3 (pan T-cell), CD4 (helper T-cell), CD8 (suppressor T-cell), CD20 (pan B-cell), CD25 (activated T-cell), CD45 (pan leukocytes), CD68 (macrophages) and Ber-Mac3 (activated macrophages) (Dakopatts, Copenhagen, Denmark) using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Cytospins made from peripheral blood leukocytes separated by a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient were processed in the same way as the sputum cells and used as a positive control.

Statistical analysis

Total and differential counts are presented as mean value (SEM). Differences between groups are analysed using the Mann–Whitney U-test. Probability values of $p < 0.05$ were considered significant. Reproducibility of sputum cell counts was examined by a repeated-measures analysis of variance with calculation of the intraclass correlation coefficient, r (the ratio of three variance of cell counts between subjects to the total variance in cell counts including observed and error variance) [12]. Values > 0.75 indicate high reliability. The inpatient variability at different time points was also assessed using the coefficient of repeatability (CR), representing the limits of agreement within which 95% of the differences are expected to be found [13]. Spearman's rank correlation (r_s) was calculated to assess the relationship between patient characteristics, lung function parameters and the cell differential count in the induced sputum COPD patients

Results

Safety and success of the method

The procedure was well tolerated by all subjects. Patients with COPD did not report adverse events nor worsening dyspnoea upon induction. In 16 out of 21 patients, spirometry was performed before and after the inhalation of hypertonic saline. No significant change in FEV₁ was seen (FEV₁ pre-inhalation: 1.22 ± 0.17 L, FEV₁ post-inhalation: 1.18 ± 0.2 L).

Reproducibility of sputum cell counts

To examine the reproducibility of sputum cell differential counts in COPD patients the intraclass correlation coefficient r was calculated. For neutrophils and macrophages, the reproducibility was high, both within investigators ($r = 0.99$ neutrophils, $r = 0.99$ macrophages, $r = 0.33$ lymphocytes, $r = 0.30$ eosinophils) and between investigators ($r = 0.95$ neutrophils, $r = 0.77$ macrophages, $r = 0.16$ lymphocytes, $r = 0.16$ eosinophils). To examine the within-subject variability, cell differential counts in two sputum samples induced at a 10-day interval were compared. The coefficients of repeatability are shown in table 2.

Table 2. – Reproducibility of cell differential count

Parameter	Week 1	Week 2	C _r	r
Total ($\times 10^3 \cdot \text{mg}^{-1}$)	23.6 (5.8)	38.4 (11.4)		
Neutrophils %	74.9 (4.7)	75.9 (4.1)	19.2	0.84
Macrophages %	20.9 (3.5)	19.1 (2.8)	17.5	0.67
Eosinophils %	2.4 (1.7)	3.1 (1.6)	3.2	0.90
Lymphocytes %	0.6 (0.2)	0.6 (0.2)	1.7	0.30
Bronchial cells %	1.1 (0.7)	1.1 (0.6)	2.3	0.91

Values are means (SEM), $n = 21$. C_r: coefficient of repeatability; r: intraclass correlation coefficient.

Differential cell counts

Patients with COPD had a higher total cell number ($23.60 \pm 5.8 \times 10^3 \text{ cells} \cdot \text{mg}^{-1}$ versus HV $5.1 \pm 0.8 \times 10^3 \text{ cells} \cdot \text{mg}^{-1}$, $p < 0.002$), an increased percentage of neutrophils (74.9 ± 4.5 versus HV 22.5 ± 4.7 , $p < 0.001$) and a decreased percentage of macrophages (20.9 ± 3.5 versus HV 74.0 ± 4.0 , $p < 0.001$). No difference was noted between COPD and HV in the percentage of lymphocytes, eosinophils, and bronchial epithelial cells. In patients with COPD, only the percentage of neutrophils correlated inversely with their FEV₁ ($r_s = -0.48$, $p < 0.05$) (fig. 1).

Immunocytochemical analysis

To further characterize the inflammatory component in COPD patients, cytospin preparations of both HV ($n = 8$) and COPD patients ($n = 8$) were used for immunocytochemistry. Analysis of cell surface markers by the APAAP technique demonstrated very few CD3+, CD4+ and CD8+ and no CD20+ or CD25+ cells. The percentage of CD45+ cells (80.5 ± 5.5 versus 52.6 ± 6.1 , $p = 0.007$) was significantly higher in COPD patients. In both groups, the majority of the macrophages (stained for CD68) were activated, as indicated by the positive staining with Ber-Mac3 (table 3).

Discussion

In this study the safety and reproducibility of sputum induction in COPD patients was examined. The cellular composition of induced sputum of COPD patients was compared with sputum from healthy volunteers. In order to avoid inadvertent bronchoconstriction reported to occur in asthmatics [14], all subjects received pretreatment with salbutamol. None of the subjects reported worsening dyspnoea nor did the spirometry values differ significantly before and after inhalation of hypertonic saline, indicating that the procedure is safe. It was also demonstrated that this method of sputum processing is reproducible as assessed

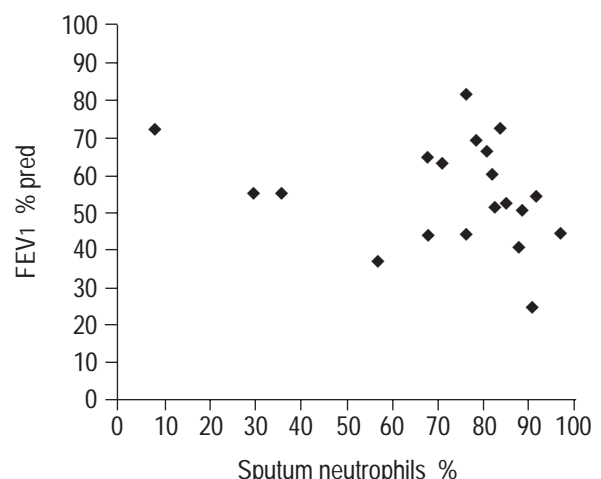


Fig. 1. – Plot of the differential counts of neutrophils (as percentages) against forced expiratory volume in one second (FEV₁) % predicted measured before sputum collection in patients with chronic obstructive pulmonary disease.

Table 3. – Cell surface markers in induced sputum as analysed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique

Parameter	COPD	Healthy
CD 3 %	1.8 (0.7)	2.1 (0.5)
CD 4 %	0.1 (0.1)	2.1 (0.2)
CD 8 %	0.3 (0.1)	0.4 (0.2)
CD 68 %	17.9 (4.8)	71.2 (4.5)*
Ber-Mac 3 %	10.1 (2.2)	47.4 (74)*
Ber-Mac3/CD68	56	66

Values are expressed as means (SEM), n=8 in both groups, calculated as percentage of nonsquamous cells. The proportion of activated macrophages is expressed as percentage of number of macrophages. COPD: chronic obstructive pulmonary disease. *: p<0.05, Mann–Whitney U-test.

by an intraclass coefficient for neutrophils and macrophages >0.75. To assess the within-subject reproducibility of induced sputum differential cell counts, sputum was induced on different occasions and a high coefficient of repeatability was found for macrophages and neutrophils only. High coefficients of reproducibility of cell counts in normal subjects have been demonstrated previously [6, 11] with intraclass correlation coefficients of >0.7 for all cell types except lymphocytes (0.3) and total cell counts (0.4). Analogous results were also demonstrated in asthmatics [15]. The methods of sputum "plug" selection used in those studies as well as in the present study may account for the improved reproducibility of total cell counts by reducing the salivary contamination. Whereas the reproducibility for counts of macrophages and neutrophils is high, poorer results are seen for lymphocytes and eosinophils. This is possibly due to the presence of much lower numbers of these cells than the abundance of macrophages and neutrophils.

In this study the cellular composition of induced sputum of COPD patients was compared with healthy volunteers. Induced sputum in COPD contains mainly neutrophils at a percentage that is almost reciprocal to that of macrophages in healthy volunteers. These observations are in line with previous findings of high percentages of intraluminal neutrophils [8, 16] and confirm previous studies demonstrating an increased number of neutrophils in bronchoalveolar lavage fluid [16, 17]. The finding that the highest neutrophil percentages were found in patients with the worst airflow obstruction is in agreement with the hypothesis that neutrophilic inflammation of the small bronchi is an important factor in the pathogenesis of airflow limitation in COPD. It has been shown that small bronchi and bronchioli are the major site of increased airflow resistance in COPD [18] and that inflammatory changes at these sites correlate with airflow limitation [19]. Assuming that induced sputum contains material from the lower airways, this technique may be a useful tool to study airway inflammation in COPD. Some discrepancies may still exist, as were shown in one study in chronic bronchitics with relatively mild airway obstruction, where the increase in the number of neutrophils and lymphocytes in the bronchial biopsies of subjects with exacerbations was not detected in sputum [9]. The disparity between low numbers of neutrophils in biopsies and high numbers in bronchoalveolar lavage and in sputum could be due to either rapid

migration through the epithelium or to a preferential peripheral location.

The excellent dispersion seen on cytospin slides allows for the application of immunocytochemical techniques. Very few CD4+ and CD8+ and no CD20+ or CD25+ cells were detected. In both groups most of the macrophages were activated as shown by the high percentage of macrophages expressing the antigen M130, recognized by the antibody Ber-Mac 3 [20]. A significantly higher number of activated macrophages was also found in bronchoalveolar lavage of asthmatics than in normal subjects [21]. However, in view of the methodological differences, the present data cannot directly be compared with this study.

In summary, the analysis of induced sputum was concluded to be a safe and reproducible method to study inflammatory cells in patients with chronic obstructive pulmonary disease. High numbers of neutrophils were found in chronic obstructive pulmonary disease and an almost reciprocal number of macrophages in healthy volunteers. A significant negative correlation was also found between sputum neutrophilia and forced expiratory volume in one second.

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