The Cellular Composition and Macrophage Phenotype in Induced Sputum in Smokers and Ex-Smokers With COPD*

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Study objectives: The relationship between smoking and COPD has been well-documented. We investigated the impact of cigarette smoking on airway inflammation in COPD patients.

Design: Changes in cell profiles in induced sputum (IS) samples from smokers with COPD and patients who ceased smoking were compared.

Setting: Department of pneumonology in a university hospital.

Patients: IS samples were collected from 17 smokers and 17 ex-smokers with COPD.

Interventions: We examined IS samples for differential cell counts and macrophage phenotypes determined by immunocytochemistry with monoclonal antibodies anti-CD11b, anti-CD14, anti-CD54, and anti-CD71.

Measurements and results: The median IS volume was greater and the total cell count was higher in smokers than in ex-smokers. The difference, however, was not significant. We did not find any significant differences in the proportions of cells and in the phenotypes of macrophages between the two groups, with the proportion of eosinophils being slightly higher in the group of smokers. We found, however, a significant positive correlation between the decrease in pulmonary function parameters and the number of pack-years smoked, an inverse correlation of pulmonary function test results with the number of lymphocytes in IS, and a correlation between some changes in the expression of macrophage surface markers and smoking history. There was no correlation between the time from smoking cessation and any cellular component found in IS samples.

Conclusions: The analysis of IS samples in patients with COPD revealed no significant differences in cell count and macrophage phenotypes between active smokers and ex-smokers.

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Key words: COPD; ex-smokers; induced sputum; macrophages; smokers

Abbreviations: AM = alveolar macrophage; BALF = BAL fluid; IS = induced sputum

COPD is a serious, progressive disorder that is characterized by poorly reversible airway obstruction and persistent inflammation in the lung. One of the major risk factors for developing COPD is cigarette smoking.1 The benefits of smoking cessation seen in the improvement in pulmonary function test results have been well-documented.1,2 By contrast, little is known about the effects of smoking cessation on the changes in the inflammatory response in the lung. The aim of this study was to investigate the effect of smoking cessation on cellular inflammation in the lungs of patients with COPD.

In the majority of studies on the cellular bases of COPD, findings from bronchial biopsy specimens, BAL fluid (BALF) samples, and induced sputum (IS) samples were analyzed. The IS sample examination is a well-tolerated, effective method for the analysis of bronchial cellular response.3–5 Several studies performed in patients with COPD revealed the role of neutrophils, macrophages, and CD8-positive lymphocytes in the pathogenesis of this disorder.6–9 Long-term smoke exposure causes similar changes in the IS cell profile with features of neutrophil and macrophage activation.10,11 It is dif-
difficult to establish the macrophage phenotype, since these cells represent a heterogeneous population with different stages of activation. The most stable surface markers seem to be the following: (1) the transferrin receptor CD71; (2) the adhesion associated molecules CD11a, CD11b, and CD11c; (3) the intercellular adhesion molecule-1 (CD54); and (4) the receptor to lipopolysaccharide-CD14.12

As shown in the investigation of the BALF from smokers, cigarette smoke alters the expression of the above markers.13,14

To investigate the differences in the airway inflammatory process in active smokers and patients who had ceased smoking, we compared the cellular composition of IS from smokers and ex-smokers with COPD. For macrophage characteristics, we used the antibodies anti-CD11b, anti-CD14, anti-CD54, and anti-CD71. The correlation between the proportion of cells in IS and the expression of macrophage markers and smoking consumption, the length of time from smoking cessation, and pulmonary function test results were analyzed.

**Materials and Methods**

**Subjects**

In this prospective study, 38 patients with COPD were included. The diagnosis of COPD was established according to the standards of the Global Initiative for Chronic Obstructive Lung Disease.1 We managed to obtain IS samples from 34 patients. The clinical characteristics of the population under study are presented in Table 1. Patients who had ceased smoking at least 1 year prior to entering the study were included in the group of ex-smokers. None of the subjects showed signs of infection during the investigation. Some of the subjects received therapy with oral steroids, with the number of treated patients and the mean dose of the steroids being comparable in the two groups of subjects. All participants gave their informed consent.

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**Table 1—Clinical Characteristics of the Study Population**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Smokers (n = 17)</th>
<th>Ex-Smokers (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, No.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Age, yr†</td>
<td>58 (46–72)</td>
<td>67 (54–82)</td>
</tr>
<tr>
<td>Duration smoking, pack-yr</td>
<td>40 (20–77)</td>
<td>40 (10–60)</td>
</tr>
<tr>
<td>Time from smoking cessation†</td>
<td>9 (1–20)</td>
<td></td>
</tr>
<tr>
<td>Treatment with OS, No. of patients</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Dose, mg/kg/4l</td>
<td>0.28 (0.22–0.50)</td>
<td>0.30 (0.12–0.45)</td>
</tr>
</tbody>
</table>

*OS = oral steroids.
†Values given as mean (range).
‡Values given as median (range).

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**Table 2—Functional Characteristics of Study Population at Baseline**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Smokers (n = 17)</th>
<th>Ex-Smokers (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1 L</td>
<td>1.21 (0.48–2.87)</td>
<td>1.43 (0.88–1.83)</td>
</tr>
<tr>
<td>% predicted</td>
<td>54 (32–74)</td>
<td>56 (49–62.5)</td>
</tr>
<tr>
<td>FVC L</td>
<td>2.4 (1.8–2.7)</td>
<td>2.2 (1.5–2.5)</td>
</tr>
<tr>
<td>% predicted</td>
<td>67 (56–90.5)</td>
<td>62 (47–76)</td>
</tr>
<tr>
<td>MEF50 % predicted</td>
<td>26 (15.5–39.6)</td>
<td>38 (14–42)</td>
</tr>
<tr>
<td>Arterial blood gas, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PaO2</td>
<td>66.3 (52.8–77.0)</td>
<td>64 (59.2–70.5)</td>
</tr>
<tr>
<td>PaCO2</td>
<td>39.7 (35.7–44)</td>
<td>39 (35.6–42.8)</td>
</tr>
</tbody>
</table>

*Values given as median (range). MEF50 = maximal expiratory flow at 50% of vital capacity.

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**Study Design**

**Sputum Induction:** All the patients were hospitalized at the Department of Pneumonology and Allergology, Warsaw Medical University. Pulmonary function tests were performed (abc PNEUMO; abcMED; Warsaw, Poland) and arterial blood gas levels were assessed prior to sputum induction. The results are shown in Table 2. The predicted values of the pulmonary function tests were taken from Knudson et al.13 The patients received 200 μg salbutamol before the induction. After undergoing spirometry after bronchodilator administration, they were asked to rinse their mouths thoroughly with water and to inhale sterile hypertonic saline solution (NaCl) at increasing concentrations (ie, solutions of 3%, 4%, and 5%) at room temperature via a pneumatic nebulizer with the output set at 0.35 mL/min. The duration of each inhalation was 5 min, and the induction was stopped after expectation of an adequate amount of sputum (ie, 2 mL). After each inhalation, spirometry was performed in order to detect a possible FEV1 decrease. The whole procedure was stopped if a 20% FEV1 decline was observed (the postbronchodilator FEV1 was considered to be the baseline value). The sputum samples obtained were transferred to the laboratory for further analysis directly after expectation.

**Sputum Processing:** IS was analyzed immediately on receipt. The volume of the sputum was measured, and the plugs were selected and weighed. A freshly prepared 0.1% solution of dithiothreitol (Sigma-Aldrich Co; St. Louis, MO) was added in a volume of a phosphate-buffered saline solution was added, and the mixture was vortexed for 15 min. Then a double volume of a phosphate-buffered saline solution was added, and the mixture was briefly vortexed. After filtration through two layers of a sterile gauze to remove debris and mucus, the sputum was centrifuged for 10 min at 800 g. The cell pellet was resuspended in a phosphate-buffered saline solution. Cell viability was determined by a trypan blue exclusion test. The cells were counted using a Bürker chamber. A differential cell count was performed using slides stained with the May-Grünwald-Giemsa method. Three hundred cells were counted using slides stained with hematoxylin-eosin. An additional two slides stained with hematoxylin-eosin were prepared for analysis of the morphology of epithelial cells.

**Immunocytochemistry:** For macrophage phenotyping, an immunocytochemistry method was used with the commercially available antibodies anti-CD14, anti-CD11b, anti-CD54, and anti-CD71 (Dako; Copenhagen, Denmark). The alkaline phos-
The proportion of macrophages, lymphocytes, neutrophils, or eosinophils in the IS between smokers and ex-smokers (Table 3). The proportion of eosinophils was slightly higher in the smokers group (median, 8%; range, 0.6 to 36.0%) than in the ex-smokers group (median, 4%; range, 0 to 28.0%), but it was not significant. There were no differences between the two groups when the differential cell count also was expressed in terms of absolute cell counts. We found a significant positive correlation between the total count of lymphocytes and the proportion of lymphocytes with FEV1 percent predicted \((r = 0.4; p < 0.05)\) and FVC percent predicted \((r = 0.4; p < 0.05)\). We found an inverse correlation of the total lymphocyte count in IS samples with arterial blood \(PCO_2\) \((r = -0.44; p < 0.05)\). We did not find any neoplastic or atypical epithelial cells in the IS smears.

The results of the analysis of macrophage surface antigens revealed no differences between the two groups (Fig 1). The intensity of reaction with antibody anti-CD71 and anti-CD11b in the cytoplasm of macrophages was stronger, but the intensity of the reaction with antibody anti-CD14 was weak.

The proportion of macrophages with the expression of CD14 correlated negatively with FVC percent predicted \((r = -0.7; p < 0.05)\). There was a reverse correlation between the proportion of macrophages with positive reaction to CD71 and the number of pack-years smoked \((r = -0.7; p < 0.05)\), and with \(PCO_2\) \((r = -0.6; p < 0.05)\). The proportion of CD11b-positive macrophages correlated with the number of pack-years smoked \((r = 0.7; p < 0.05)\) and with \(PCO_2\) \((r = 0.6; p < 0.05)\).

Table 3—Total and Differential Cell Counts in IS Samples of Study Groups*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Smokers (n = 14)</th>
<th>Ex-Smokers (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count, (\times 10^6) cells/mL</td>
<td>4.8 (2.1–10.4)</td>
<td>3.0 (1.1–5.5)</td>
</tr>
<tr>
<td>Squamous cells, † %</td>
<td>28.0 (10–30.5)</td>
<td>17.0 (10.0–23.0)</td>
</tr>
<tr>
<td>Macrophages ‡ % (\times 10^6) cells/mL</td>
<td>26.5 (11.0–47.0)</td>
<td>35.0 (17.0–39.0)</td>
</tr>
<tr>
<td>Lymphocytes ‡ % (\times 10^6) cells/mL</td>
<td>1.26 (0.74–2.58)</td>
<td>1.32 (0.83–1.97)</td>
</tr>
<tr>
<td>Neutrophils ‡ % (\times 10^6) cells/mL</td>
<td>2.2 (1.0–4.0)</td>
<td>1.5 (0.8–3.0)</td>
</tr>
<tr>
<td>Eosinophils ‡ % (\times 10^6) cells/mL</td>
<td>0.10 (0.04–0.23)</td>
<td>0.06 (0.03–0.17)</td>
</tr>
</tbody>
</table>

*Values given median (25th to 75th percentile).
† Expressed as the percentage of all cells.
‡ Expressed as the percentage of nonsquamous cells.
We did not find any correlation between the proportion of cells or macrophage markers and the length of time from smoking cessation. There was no correlation of the mean dose of steroids with any of the investigated parameters.

**Discussion**

This study showed that there are no significant differences in the cellular profile of IS samples between patients with COPD who are active smokers and those who have ceased smoking. To the best of our knowledge, ours is the first comparison between these groups of patients. In the majority of studies on IS samples from patients with COPD, what was compared was the sputum samples received from healthy subjects or a group of smokers with those from nonsmokers. There have been few studies of the effect of smoking cessation on cellular changes in the airways. Skold et al\(^{16}\) presented the results of BALF analyses in healthy persons, Turato et al\(^{17}\) showed the results of examinations of bronchial biopsy specimens in patients with chronic bronchitis, and Wright et al\(^{18}\) found similar structural changes in the lungs of smokers and ex-smokers with emphysema. In these investigations, no differences in the changes in the cellular profiles of smokers vs ex-smokers were found.

In this study, the clinical characteristics of the groups of smokers and ex-smokers did not differ significantly. We found a significant inverse correlation between the results of pulmonary function tests, and we found a positive correlation between the arterial blood \(\text{PCO}_2\) values and smoking consumption, which were expressed as the number of pack-years in both groups that were analyzed. The group of ex-smokers was composed of patients who had ceased smoking \(>1\) year earlier, and the maximum time from smoking cessation was 20 years. We did not find any correlation between the results of pulmonary function tests and the length of time from smoking cessation.

The effectiveness of sputum induction was comparable in both groups. The mean volume of sputum and the total cell count were higher in active smokers when compared with those of ex-smokers and was close to normal values in healthy subjects. Skold et al\(^{16}\) found that the normalization of the total cell count and the cell concentration in the BALF samples to be in proportion to the length of time from smoking cessation.

No significant differences were found when the comparison of total and differential cell counts was made between the groups. The differential cell count in IS samples from our patients with COPD was similar to those reported by other authors.\(^{19-23}\) However, in other investigations the percentage of neutrophils in smokers with COPD was higher than in our patients,\(^{21-23}\) but the number of neutrophils was similar.\(^{22}\) We noted an elevated proportion of eosinophils and higher total number of eosinophils in both smokers and ex-smokers with COPD, and a slightly higher proportion of these cells in smokers when compared with ex-smokers (difference not significant). In the study of Hargreave and Leigh,\(^{20}\) the participation of eosinophils in bronchial constriction in smokers was observed. Lebowitz et al\(^{24}\) concluded that eosinophilia is an important aspect of bronchial

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**Figure 1.** Macrophage phenotype in the IS samples of the study groups. The data are shown as the median (p25-p75) percentage of macrophages. sm = smokers; ex = ex-smokers.
constriction in smokers. Cosio and Guerassimov\textsuperscript{25} divided case of COPD in smokers into the following two types: one with eosinophilia; and the other without eosinophilia. There were no differences in IS or BAL samples in the proportion of eosinophils between healthy smokers and nonsmokers in the study of Lensmar et al.\textsuperscript{11} It may be that eosinophilia found in IS samples may reflect the presence of inflammation in smokers with COPD who have airway responsiveness to corticosteroids, which is an asthmatic pattern of COPD.\textsuperscript{8} A slightly lower proportion of eosinophils observed in IS samples from ex-smokers may indicate a normalization of the inflammatory process in the airways after smoking cessation.

Pulmonary macrophages are a heterogeneous population of cells. Most studies have been concerned with alveolar macrophages (AMs) obtained by BAL of the peripheral airways. The cells in IS samples originate from the proximal airways, and it has been shown that macrophages in IS samples represent a more mature phenotype.\textsuperscript{3} However, it is possible to find an expression of the same markers on IS macrophages as that of the same markers on the AMs present in the BALF.\textsuperscript{11} For the analysis of pulmonary macrophages, we used antibodies recognizing some of the most stable surface markers, such as CD11b, CD14, CD54, and CD71.\textsuperscript{12} The proportion of CD54-positive macrophages and the proportion of CD71-positive macrophages in IS samples from active smokers with COPD in our study were lower than those in the group of healthy smokers reported by Lensmar et al.,\textsuperscript{11} but the proportion of macrophages with expression of CD11b was higher in our study than in theirs.\textsuperscript{11} Long-term exposure to cigarette smoke has been shown to alter the expression of these markers. The proportion of CD11b-positive, CD14-positive, CD54-positive, and CD71-positive macrophages was lower in the BALF and IS samples of smokers when compared with those of nonsmokers.\textsuperscript{11,20,27} In the study of Schaberg et al.,\textsuperscript{13} the increased expression in CD11/CD18 on AMs from smokers was found. In our analysis, the proportion of macrophages expressing CD11b, CD14, CD54, and CD71 did not differ significantly between smokers and ex-smokers with COPD.

CD11b seems to be an important agent in eosinophil activation.\textsuperscript{28} We found a slightly elevated proportion of eosinophils in current smokers, but there was no correlation with the proportion of CD11b-positive macrophages. A positive correlation between CD11b macrophages and smoking history, expressed as the number of pack-years smoked, and blood $\text{PCO}_2$ that was observed in our study may reflect the role of this molecule in the pathogenesis of COPD in smokers. This was also a hypothesis put forward by Saetta et al.\textsuperscript{8} We found a correlation between the proportion of macrophages expressing CD14 and FVC, and between the proportion of macrophages expressing CD71 and arterial $\text{PCO}_2$. The role of these cells in the structural changes that occur in COPD has not been elucidated. In the study of Turato et al.\textsuperscript{17} on the influence of smoking cessation on cellular changes found in bronchial biopsy specimens from COPD patients, no changes in macrophage count were found.

We have not found any differences in the cellular profiles and macrophage phenotypes of patients with COPD, when comparing smokers with ex-smokers. It may be that smoking causes persistent inflammatory changes in the airways in patients who are predisposed to COPD.

References