The aim of our study was to evaluate cellular content in induced sputum in chronic obstructive pulmonary disease (COPD) in relation to the degree of airway obstruction, macrophage count, and phenotype. We compared the proportion of macrophages and cells expressing the following markers: CD11b, CD14, CD54, and CD71 in induced sputum obtained from patients with mild-to-moderate and severe COPD (n=29), asymptomatic smokers (n=18), and nonsmokers (n=18). The differential cell count and macrophage phenotypes were examined in induced sputum by immunocytochemistry. We observed a greater proportion of neutrophils and eosinophils and an elevated macrophage count in patients with COPD and in smokers in comparison with nonsmokers. Macrophages in patients with severe airway obstruction were characterized by a significantly elevated expression of CD11b and CD14 markers. There were higher proportions of macrophages with expression of CD11b, CD14, CD54, and CD71 in induced sputum of smokers in comparison with nonsmokers. We concluded that macrophages are the cells involved in the inflammatory process caused by smoking in COPD. The macrophage phenotype with elevated CD11b and CD14 expressions was associated with severe airflow limitation.

Key words: COPD, induced sputum, macrophages

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an increasing global health problem (1). Smoking plays a major role in the pathogenesis of this disease, but the reasons why only 15-20% of smokers develop airflow limitation and why the disease occurs also in non-smokers remain unclear. Except for smoking history,
little is known about the prognostic factors and thus investigating the inflammatory status of smokers with and without COPD seems justified (2). Induced sputum (IS) examination is an appropriate method of the evaluation of airway inflammation in COPD (3-5). In the pathogenesis of COPD the participation of macrophages, among other cellular components of inflammation, was postulated (2, 4, 6-8). The aim of our study was to evaluate changes in the cellular content in IS in COPD in relation to the degree of airway obstruction and to compare these changes with those observed in smokers without airflow limitation and healthy non-smokers with particular attention to macrophage count and phenotype. To characterize the population of macrophages we chose the following surface markers: CD11b, CD14, CD54, and CD71. These markers reflect the involvement in the inflammatory process by cell-to-cell contact and cell activation, and are related to the impact of tobacco smoke (4, 9-11).

MATERIAL AND METHODS

Subjects

Twenty nine patients with COPD, 18 asymptomatic smokers and 18 healthy non-smokers were included in the study. The diagnosis of COPD was established according to the ATS/ERS

<table>
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<th>Table 1. Clinical characteristics of the study population.</th>
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Data are shown as median (p25- p75).
standards (1). We divided the group of patients into those with mild/moderate COPD - FEV1 ≥ 50% predicted (n = 18) and those with severe disease - FEV1 < 50% predicted (n = 11). Clinical characteristics of the study population are presented in Table 1. None of the subjects had symptoms of infection or exacerbation of the disease during the investigation. All the patients and healthy volunteers gave their informed consent to participate in the study. The investigation was approved by the Ethics Committee of the Warsaw Medical University in Warsaw, Poland.

Sputum induction

Pulmonary function tests were performed (abcPNEUMO, abcMED, Poland) and arterial blood gases were assessed prior to sputum induction (Table 2). The patients received 200 µg of salbutamol before the induction. After the postbronchodilator spirometry they inhaled sterile hypertonic saline (NaCl) at increasing concentrations (3%, 4%, and 5% solutions) 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 expectoration of an adequate amount of sputum (2 ml). After each inhalation spirometry was performed in order to detect a possible FEV1 decrease. The whole procedure was stopped when a 20% FEV1 decline was observed.

Sputum processing

Induced sputum was analyzed immediately on receipt as previously described (12, 13). The volume of the sputum was measured. A freshly prepared 0.1% solution of dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO) was added in the volume equal to the double weight of the sputum, and the mixture was vortexed for 15 min. Then a double volume of a phosphate buffered solution (PBS) was added and the mixture was briefly vortexed. After filtration the sputum was centrifuged for 10 min at 800 x g. The cell pellet was resuspended in PBS. The cells were counted using a Bürker chamber. Differential cell count was performed on slides stained with the May-Grunwald-Giemsa method. Three hundred cells were counted. Smears with less than 50% of squamous cells and more than 200 nonsquamous cells were qualified as adequate.

For macrophage phenotyping an immunocytochemical method was used with commercially available antibodies anti: CD14, CD11b, CD54, and CD71 (Dako, Denmark). The alkaline phosphatase anti-alkaline phosphatase (APAAP) reaction was performed on the air dried slides according to the instruction of the manufacturer (LSAB2 kit, Dako, Denmark). In the first step the slide was covered by primary antibody and negative control reagent. Next Link was added and incubation with streptavidin-alkaline phosphatase was performed for 10 min. The freshly prepared substrate chromogen-solution was added for 10 min and after rinsing with distilled water slides were counterstained with Mayer's hematoxylin. Two hundred macrophages on each reaction area were evaluated under a light microscope and the percentage of positive cells was recorded.

Statistical analysis

Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Statistical significance was set at P<0.05. The relationships between the proportion of cells and smoking history, expressed as pack per years, and the results of pulmonary function tests were analyzed with the Spearman correlation coefficient. Correlations with both r ≥ 0.4 and P<0.05 were considered relevant.
RESULTS

No clinical signs of bronchoconstriction during sputum induction were observed. The mean volume of the sputum obtained was 3.5 ml and it was comparable in all the three investigated groups. The mean cell viability was higher than 90%. The total cell count was elevated in the group of patients with COPD (median 3.8, range 0.3-52.0 x 10^6). In the group of asymptomatic smokers the median total cell count was 1.7, range 0.3-8.2 x 10^6, and in healthy nonsmokers it was 1.3, range 0.2-41.0 x 10^6. Sputum of patients with mild/moderate COPD was characterized by a higher total cell count compared with patients with severe COPD (median values were 4.2 x 10^6 and 3.0 x 10^6, respectively).

The differential cell count is presented in Table 3. We found a significantly higher proportion of macrophages in healthy smokers and nonsmokers compared with COPD patients. Proportions of lymphocytes and of neutrophils did not differ between the investigated groups. Induced sputum of COPD patients had an elevated number of macrophages and neutrophils (significant differences when compared with healthy nonsmokers). The highest number of macrophages was found in patients with mild/moderate airway obstruction and that of neutrophils in patients with severe airflow limitation. The percentage of eosinophils was elevated in COPD patients, especially in those with severe airway obstruction; the difference was significant compared with healthy subjects. It also is noteworthy that asymptomatic smokers had a significantly higher proportion of IS eosinophils compared with healthy nonsmokers.

An analysis of macrophage surface antigens revealed some significant differences between the study groups (Figures 1 - 4). The intensity of reaction with antibodies against CD71, CD54, and CD11b in the cytoplasm of macrophages was strong, but that with anti CD14 antibody was weak. There were

| Table 3. Differential cell count as a percent of nonsquamous cells in induced sputum of study groups. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                | COPD Patients                   | Asymptomatic Smokers            | Healthy Nonsmokers              |
|                                | (n=29)                          | (n=18)                          | (n=18)                          |
|                                | Mild/moderate                   | Severe                           |                                  |
|                                | airway obstruction              | airway obstruction               |                                  |
|                                | (n=18)                          | (n=11)                          |                                  |
| Macrophages ×10^6 %             | 31 (16-45)†                     | 29 (17-46)                      | 33 (16-39)                      |
|                                | 1.3 (0.8-2.6)                   | 1.4 (0.9-2.6)                   | 0.95 (0.77-2.65)                |
| Lymphocytes ×10^6 %             | 2 (1-5)                         | 2.5 (1-5)                       | 1.7 (1.8-4.5)                   |
|                                | 0.09 (0.04-0.29)                | 0.13 (0.06-0.36)                | 0.04 (0.03-0.10)                |
| Neutrophils ×10^6 %             | 54 (37-68)†                     | 54 (35-70)                      | 51 (39-60)                      |
|                                | 2.1 (1.0-5.6)                   | 2.1 (0.98-7.8)                  | 2.3 (1.15-3.96)                 |
| Eosinophils ×10^6 %             | 5.0 (2.4-21)†                   | 4.5 (2.5-23)                    | 6 (0.6-21)                      |
|                                | 0.42 (0.05-1.25)†               | 0.44 (0.11-0.95)                | 0.35 (0.03-1.99)                |

Data are shown as median (p25- p75). #, †, *- P<0.05 in the corresponding group comparison.
no differences in reaction intensity with the above mentioned antibodies between the study groups. IS of smokers had an elevated proportion of all analyzed markers compared with healthy nonsmokers. Patients with severe airflow

*Fig. 1.* Expression of CD14 on macrophages in IS of the study groups. Data are shown as median (p25-p75) percentage of macrophages. The proportion of CD14 positive macrophages in patients with severe bronchial obstruction is significantly higher than in patients with mild obstruction and in asymptomatic smokers.

*Fig. 2.* Expression of CD11b on macrophages in IS of the study groups. Data are shown as median (p25-p75) percentage of macrophages. There is a significant difference in the proportion of CD11b macrophages in patients with severe COPD compared with healthy nonsmokers.

*Fig. 3.* Expression of CD71 on macrophages in IS of the study groups. Data are shown as median (p25-p75) percentage of macrophages. No significant differences were found.
limitation had a significantly higher proportion of macrophages expressing CD14 and CD11b and a lower proportion of those expressing CD71 and CD54 compared with patients with mild/moderate airway obstruction and healthy smokers. We also observed an inverse correlation of FEV1 % predicted with the total cell (r = -0.32, P<0.03) and neutrophil counts (r = -0.43, P<0.05). The mean number of pack/years smoked correlated with the proportion of CD11b positive macrophages (r = 0.55, P<0.05).

DISCUSSION

In this study we investigated the IS obtained from patients with COPD and healthy subjects. We focused on the macrophage surface markers that reflect cell activation and upregulation by interaction with other inflammatory cells known to participate in the pathogenesis of airflow limitation. We analyzed the study population in relation to smoking history and the degree of airway obstruction. We demonstrated changes in the population of macrophages in IS of COPD patients. While the proportion of macrophages was lower in COPD patients compared with healthy subjects, the absolute number of these cells was higher in the former group. Usually, in the quantitative analysis of IS or bronchoalveolar lavage fluid (BALF) the differential cell count is presented as a proportion of cells, whereas the cell number may reflect the character and type of inflammation more appropriately and may facilitate comparative studies, e.g., with the results of bronchial biopsies (2, 4, 10). Participation of macrophages in the airway inflammation of COPD was postulated in many recent a study. Di Stefano et al (6) observed a correlation of severity of COPD and FEV1 decline with the number of macrophages infiltrating bronchial subepithelium. This infiltration was greater in COPD patients than in asymptomatic smokers. IS represents large airways and our findings are in agreement with those of Di Stefano et al (6).
COPD is a complicated disorder and emphysema is one of the components of this disease (1). Finkelstein et al (14) documented the influx of macrophages into emphysematous lungs. One of the suspected effects of macrophages in the pathogenesis of emphysema and COPD in smokers is an elastolytic and proteolytic activity and enhancement of the inflammatory process by increased secretion of cytokines (7, 15). Comparing asymptomatic smokers with healthy nonsmokers, a higher macrophage count in smokers was observed in our study, which accords with the results of D'Ippolito et al (16) and Willemse et al (17).

Our study confirmed previous observations that the number of neutrophils in IS correlates with the severity of airway obstruction in smokers (7, 18, 19). Additionally, we found that the proportion of eosinophils also was higher in such patients. An elevated eosinophil proportion in smokers with airway obstruction has been reported before and the type of COPD with asthma-like features has been postulated (7, 13, 20, 21). Balzano et al (22) found a significant correlation between the decline of FEV₁ and the proportion of eosinophils in smokers with and without COPD. In the study of D'Ippolito et al (16) the eosinophil count was higher in smokers when compared with nonsmokers. Likewise, we found a significant difference in the proportion and absolute number of eosinophils between healthy smokers and nonsmokers.

Having confirmed the changes in the macrophage count, we analyzed the expression of selected surface markers on macrophages. Little is known about the macrophage phenotype in IS samples in COPD. In our study the relation of these markers with the degree of airflow limitation was investigated. In healthy nonsmokers the proportion of macrophages expressing all markers investigated was significantly lower than that in smokers (with and without COPD). This proportion was lower than that in the studies of Lensmar et al (23, 24), but higher than that reported by Maestrelli et al (25). Striz et al (26) showed that the phenotype of alveolar macrophages in BALF reflects the immunologic status of the lung and the interactions with other lymphoid cells. However, sputum macrophages differ from alveolar ones morphologically, which should be taken into account when comparing various studies.

In this study we investigated the expression of CD14, the receptor for lipopolisaccharides binding protein. This marker is expressed to a higher degree in blood monocytes than in tissue macrophages (27, 28). The CD14 plays a role in macrophage activation in infection and other inflammatory processes (29). Le Barillec et al (29) described the human leukocyte elastase as an inhibitor of CD14. Neutrophil elastase is a key agent in the pathogenesis of COPD (30) and that macrophages inhibit neutrophil elastase release by phagocytosis of apoptotic neutrophils (31), which emphasizes the role of membrane CD14 in the inflammation of COPD. We found a significantly increased proportion of CD14 positive macrophages in IS of patients with severe airway obstruction, which possibly reflects the influx of young mononuclear cells into the bronchial lumen. Similar results were reported by Frankenberger et al (32). The proportion of
CD14 positive macrophages in our study was higher in smokers than in nonsmokers. The effect of cigarette smoke on CD14 expression by alveolar macrophages may be caused by an elevated concentration of lipopolisaccharides binding protein in the particulate phase of tobacco smoke (33).

CD11b represents the integrin family and is known to be overexpressed on neutrophils in COPD (30). Participation of adhesion molecules was postulated in the pathogenesis of COPD and in the effects of tobacco smoke on the lung tissue (4, 30, 34). We found an elevated proportion of CD11b positive macrophages in patients with severe COPD, which correlated with the decline in FEV₁ (data not shown). This observation supports the role of cells recruited from circulation in the pathogenesis of COPD progression, as blood monocytes have a higher expression of CD11b than alveolar macrophages (23, 24). In contrast, Gonzalez et al (34) reported that airway obstruction cannot be explained by differences in the expression of adhesion molecules (34). Tobacco smoke alters the expression of integrins on macrophages. In the study of Schaber g et al (35) the expression of CD11/CD18 on alveolar macrophages was higher in BALF of smokers. In our study the expression of CD11b significantly correlated with the number of pack/years smoked.

No significant differences were found in the proportion of CD54 and CD71 positive macrophages between the patients with mild/moderate and severe airflow limitation, apart from an elevated expression of these markers in IS of smokers compared with nonsmokers. This finding is in agreement with the results of Sköld et al (11), but is opposite to those of Lensmar et al (24). In our study the proportion of CD54+ macrophages was relatively lower in smokers with airway obstruction than in asymptomatic smokers. Recently, Wehlin et al (37) reported lack of changes in the expression and mobilization of adhesion molecules on blood leucocytes and monocytes of healthy smokers and patients with COPD.

In conclusion, an elevated proportion of IS macrophages expressing CD14 and CD11b and lower of those expressing CD54 and CD71 in relation to airway obstruction may indicate the influx of young mononuclear cells and alterations in their activation. Smoking seems influential for affecting the macrophage phenotype in IS. The interaction of macrophages with other lymphoid cells via adhesion molecules in the pathogenesis of COPD needs further investigation.

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