Inflammatory and immunological profiles in patients with COPD: relationship with FEV$_1$ reversibility

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ABSTRACT

Objective: To determine whether COPD severity correlates with sputum cell counts, atopy, and asthma. Methods: This was a cross-sectional study involving 37 patients with COPD and 22 healthy subjects with normal lung function (controls). Sputum cell counts were determined by microscopy after centrifugation of samples. Skin prick tests were performed, and serum cytokines were determined by ELISA. Results: Patients were stratified by bronchodilator response: a non-reversible airflow limitation (nonsmoker or RAL) group comprised 24 patients showing no significant post-bronchodilator change in FEV$_1$; and a partially reversible airflow limitation (partially reversible bronchodilator or RAL) group comprised 13 patients showing FEV$_1$ reversibility (post-bronchodilator FEV$_1$ increase ≥ 12%). The proportion of eosinophils in sputum was higher in the nonsmoker or RAL group than in the partially reversible bronchodilator or RAL group (p < 0.01), and there was an inverse correlation between the proportion of eosinophils and FEV$_1$ (p < 0.05). However, none of the patients had a history of asthma and skin prick test results did not differ between the two groups. In the patient sputum samples, neutrophils predominated. Serum levels of TNF, IL-6, IL-8, and RANTES (CCL5) were higher in patients than in controls (p < 0.001) but did not differ between the two patient groups. Conclusions: COPD patients with partial FEV$_1$ reversibility appear to have higher sputum eosinophil counts and greater airway hyperresponsiveness than do those with no FEV$_1$ reversibility. However, we found that COPD severity did not correlate with atopy or with the cytokine profile. Keywords: Pulmonary disease, chronic obstructive; Cytokines; Chemokines; Eosinophils; Sputum/cytology; Forced expiratory volume.

INTRODUCTION

COPD is an inflammatory disorder that affects the airways, pulmonary parenchyma, and pulmonary vessels, progressing slowly to irreversible airway obstruction. Although studies have shown that neutrophil and eosinophil counts are both elevated during COPD exacerbations, neutrophilic inflammation is the norm in COPD.$^{(4)}$ However, even in the stable phase of the disease, eosinophils are found in up to 40% of patients.$^{(2)}$ It is thought that this feature is related to a COPD subtype—COPD with asthma, also known as the asthma–COPD overlap syndrome.$^{(2)}$ Patients with that syndrome show FEV$_1$ reversibility after bronchodilator use.

The inflammatory response of the airways has received special attention in recent years.$^{(3,4)}$ Levels of inflammatory mediators such as C-reactive protein, IL-8, IL-6, TNF, and RANTES (CCL5) have been found to be elevated in COPD.$^{(5-7)}$ In addition, neutrophil counts are higher in smokers with COPD, as are levels of IL-8 and eosinophil cationic protein.$^{(4)}$ According to Lapperre et al.,$^{(8)}$ it is possible that inflammation associated with smoking occurs in two stages: an initial phase, during which neutrophils and macrophages are present in the epithelium and submucosa, and a late stage, with the additional participation of lymphocytes and eosinophils. Nevertheless, the association between COPD and asthma has been controversial, and the influence that eosinophils have on airway inflammation and on the severity of COPD is not completely understood.

The severity of airflow obstruction can be determined by quantifying the magnitude of the decrease in FEV$_1$, and the stages of COPD are based on the post-bronchodilator FEV$_1$.$^{(10)}$ Once the diagnosis of COPD has been made, pulmonary function tests are useful for quantitative monitoring of the course of the disease. To determine the severity of COPD, FEV$_1$, and its reversibility are reconciled with the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification.$^{(10)}$ The aim of the present study was to determine whether COPD severity correlates with FEV$_1$ reversibility, asthma, and atopy. In addition, we evaluated the relationship between serum cytokine levels and COPD subtypes based on FEV$_1$ reversibility.

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**METHODS**

This was a cross-sectional study involving 37 patients diagnosed with COPD on the basis of the GOLD criteria. All patients were under treatment in the Pulmonology Department of Professor Edgard Santos University Hospital, in the city of Salvador, Brazil. The study was approved by the Research Ethics Committee of the Hospital (Protocol no. 113/2012), and all participating patients gave written informed consent. All of the patients completed questionnaires designed to evaluate the history of asthma in childhood, smoking, passive smoking, and allergic rhinitis. All patients also underwent physical examinations and pulmonary function tests with an emphasis on the functional parameters FEV$_1$, FVC, and FEV$_1$/FVC ratio. According to the GOLD criteria, an FEV$_1$/FVC ratio ≤ 70% of the predicted value is diagnostic of COPD. Spontaneous or induced sputum samples were obtained, skin prick tests were performed in order to assess sensitivity to allergens, and 10-mL blood samples were collected for determination of serum cytokine levels. A group of 22 healthy subjects without COPD (with normal lung function) were used as controls.

On the basis of the pulmonary function test parameters established in the 2010 GOLD guidelines and the response to bronchodilator, the COPD patients were divided into two groups: non-reversible airflow limitation (non-RAL), comprising the patients who showed no significant post-bronchodilator change in FEV$_1$ (n = 24); and partially reversible airflow limitation (partial-RAL), comprising the patients who showed FEV$_1$ reversibility (n = 13). Post-bronchodilator FEV$_1$ reversibility was defined as a ≥ 12% increase in FEV$_1$, as proposed in the joint American Thoracic Society (ATS)/European Respiratory Society (ERS)/GOLD guidelines. The control group was composed of (N = 22) healthy subjects without COPD and with normal lung function.

All patients underwent spirometry in accordance with the ATS/ERS/GOLD joint guidelines. Bronchodilator testing was performed with 100 µg/mL of albuterol sulfate (Aeroli® [Ventolin®]; GlaxoSmithKline Brasil Ltda., Rio de Janeiro, Brazil). In brief, four puffs (400 µg/mL) were administered with the aid of a spacer (Fumax®; GlaxoSmithKline Brasil Ltda.). At 15 min after administration of the bronchodilator, the pulmonary function tests were repeated.

Patients were submitted to the immediate hypersensitivity skin prick tests, as described by Pepys et al., and modified by Osterbalee and Weeke. The allergens tested included dog dander, cat dander, airborne fungi (Aspergillus fumigatus), cockroach allergens (from Blattella germanica and Periplaneta americana), and dust mite allergens (from Dermatophagoides pteronyssinus and Blomia tropicalis). Reagents were obtained from ImmunoTech (a division of FDA Allergenic Ltda., Rio de Janeiro, Brazil).

Sputum induction was performed in accordance with the modified protocol described by Pavord et al., with inhalation of hypertonic saline solution (3%, 4%, and 5%) in an ultrasonic nebulizer (Fisoneb®; Canadian Medical Products, Ltd, Markham, Ontario, Canada) at a low flow rate (0.87 L/min). Peripheral blood samples (10 mL each) were centrifuged at 2,000 rpm for 10 min. Serum was collected and stored at −20°C for subsequent cytokine measurements. Cytokines and chemokines were quantified by sandwich ELISA according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA). To quantify TNF and IL-6, we used high sensitivity kits (Quantikine HS ELISA; R&D Systems). To quantify RANTES (CCL5) and IL-8, we used DuoSet ELISA kits (R&D Systems).

In the statistical analysis, we used measures of central tendency, including means and medians, for demographic and clinical variables. Data were analyzed using the Statistical Package for the Social Sciences, version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The values obtained for the variables body mass index, SpO$_2$, and lung function, which typically have a normal distribution, were analyzed with Student’s t-tests. For the comparison between sputum cell counts and the stages of COPD severity, we used the Mann-Whitney test. The correlation between eosinophils in sputum and FEV$_1$ (before and after bronchodilator use) was analyzed by Spearman’s correlation coefficient. The comparisons among the non-RAL, partial-RAL, and control groups, in terms of cytokine production, sputum cell counts, and COPD severity, were made with the Kruskal-Wallis test, followed by Dunn’s post-test for multiple comparisons.

**RESULTS**

**Characteristics of the sample**

The demographic features, smoking status, and pulmonary function test results of the COPD patients, by group (with or without post-bronchodilator FEV$_1$ reversibility), are shown in Table 1. There were no differences between the two patient groups regarding age, gender, or smoking status. There were also no differences between the two groups in terms of the body mass index, SpO$_2$, or age at the onset of symptoms. All patients in the sample had an FEV$_1$/FVC ratio ≤ 70% of the predicted value. The median values for pre- and post-bronchodilator FEV$_1$ were 48.2% (range, 30-66%) and 51% (range, 35-71%), respectively, in the non-RAL group, compared with 35% (range, 28-44%) and 47% (range, 36-52%), respectively, in the partial-RAL group (p < 0.04), whereas they were 79% (range, 65-82%) and 84% (range, 69-89%), respectively, in the control group. None of the control subjects were smokers or former smokers.

**COPD severity, sputum cell counts, and atopy**

As can be seen in Table 2, the severity of COPD was assessed in accordance with the 2010 GOLD guidelines. The 24 patients in the non-RAL group were fairly equally distributed among the COPD stages II, III, and IV, whereas 12 (92.3%) of the 13 patients in...
the partial RAL group were categorized as having stage III COPD. Although neutrophil counts increased in proportion with the severity of COPD, eosinophils were not detected in patients with stage IV COPD (data not shown).

In both patient groups, we performed differential counts of neutrophils and eosinophils in the sputum samples (Table 2). The neutrophil counts did not differ between the two groups (p > 0.05). However, the median eosinophil count was significantly higher in the partial RAL group than in the non RAL group (p < 0.01).

In our evaluation of atopy, with the skin prick test, we found no difference between the two patient groups in terms of positivity for any of the antigens tested (Table 2). Of the 24 patients in the non RAL group, 5 (21%) had at least one positive test, compared with 4 (31%) of the 13 patients in the partial RAL group (p > 0.05). Moreover none of the patients in either patient group had a history of asthma. In 2 of the patients in the non RAL group, the response to histamine was negative.

**Relationship between the proportion of eosinophils and FEV₁**

The relationship between the proportion of eosinophils in the sputum and FEV₁ (before and after bronchodilator use) is shown in Figure 1. There was an inverse relationship between the proportion of eosinophils in the sputum and FEV₁ before and after bronchodilator use (p < 0.01).

**Table 1.** Demographic, clinical, and pulmonary function characteristics of patients with COPD.*

| Characteristic                          | Group         | p*     |
|----------------------------------------|---------------|--------|
| Gender, n (%)                          |               |        |
| Male                                   | non RAL (n = 24) | 11 (45.8) | 6 (46.2) | 0.72 |
|                                        | partial RAL (n = 13) | 13 (54.2) | 7 (53.8) |        |
| Age, years                             |               | 66 (57-73) | 69 (59-76) | 0.88 |
| Smoking, n (%)                         |               | 8 (27.3) | 4 (30.8) | 0.67 |
| Former smokers, n (%)                  |               | 16 (72.7) | 9 (69.2) | 0.43 |
| Body mass index, kg/m²                 |               | 22 (19-25) | 18 (18-21) | 0.001 |
| SpO₂, %                                |               | 96 (94-97) | 95 (93-97) | 0.93 |
| Age at symptom onset, years            |               | 54 (42-58) | 55 (43-56) | 0.97 |
| FEV₁/FVC ratio, % of predicted         |               | 64 (53-70) | 59 (53-69) | 0.44 |
| Pre-BD                                 |               | 66 (51-62) | 62 (57-68) |        |
| Post-BD                                |               | 48 (30-66) | 35 (28-42) | 0.04 |
| FEV₁, % of predicted                   |               | 51 (35-71) | 40 (35-50) |        |
| Pre-BD                                 |               | 69 (57-84) | 58 (53-66) | 0.06 |
| Post-BD                                |               | 76 (64-89) | 69 (59-77) |        |

*Student’s t-test (two-tailed); level of statistical significance set at p < 0.05.

**Table 2.** Cell counts in induced sputum samples, by COPD stage, and results of allergy testing in patients with COPD, by group.*

| Variable                              | Group         | p     |
|---------------------------------------|---------------|-------|
|                                      | non RAL (n = 24) | partial RAL (n = 13) |     |
| GOLD stage                            |               |       |
| II                                    | 6 (27.3) | 0 (0.0) | 0.03 |
| III                                   | 12 (45.5) | 12 (92.3) | 0.77 |
| IV                                    | 6 (27.3) | 1 (7.7) | 0.43 |
| Skin prick test                       |               |       |
| Positive                              | 5 (20.8) | 4 (30.8) | 0.58 |
| Negative                              | 17 (70.8) | 9 (69.2) | 0.09 |
| Proportion of neutrophils*            | 38 (24-52) | 37 (26-47) | 0.67 |
| Proportion of eosinophils*            | 0 (0-7) | 9 (2-15) | 0.01 |

*non RAL: non-reversible airflow limitation; partial RAL: partially reversible airflow limitation; and BD: bronchodilator. *Values expressed as number (%); **Values expressed as median (interquartile range). **Mann-Whitney test (two-tailed); level of statistical significance set at p < 0.05.
**Immunological profile**

The cytokine and chemokine levels in the non-RAL group, partial-RAL group, and control group are shown in Figure 2. The median TNF levels were 2.9 pg/mL (range, 0.95-6.03 pg/mL) in the non-RAL group and 3.2 pg/mL (range, 2.65-5.50 pg/mL) in the partial-RAL group, both of which were significantly higher than the 0.35 pg/mL (range, 0-1.9 pg/mL) observed for the controls (p < 0.01). The median IL-6 levels were also significantly higher in the partial-RAL group and RANTES (CCL5) levels did not differ significantly between the two patient groups or between either patient group and the control group (p > 0.05 for all). As depicted in Figure 2, the median IL-8 values for the non-RAL, partial-RAL, and control groups were 0 (range, 0-57.50), 0 (range, 0-51.75), and 0 (range, 0-0), respectively, the difference between the non-RAL group and the control group being statistically significant (p < 0.05). There were no statistical differences among the three groups in terms of the serum levels of RANTES (CCL5; Figure 2). The severity of COPD did not correlate significantly with the serum levels of TNF, IL-6, IL-8, or RANTES (CCL5; data not shown).

**DISCUSSION**

COPD is a severe progressive inflammatory disease and is the fourth leading cause of death in the United States. ([16]) The prevalence of and mortality associated with COPD continue to rise. In addition, COPD has become a major cause of death and disability worldwide. ([17]) Two COPD subtypes, based on FEV \(_1\) reversibility, have recently been identified. Here, we attempted to determine whether those subtypes are associated with sputum cell counts, cytokine levels, and symptom severity. We found that COPD patients with reversibility of FEV \(_1\) had higher sputum eosinophil counts and greater airway hyperresponsiveness than did those without such reversibility, and that there was an inverse correlation between the proportion of eosinophils in the sputum and the FEV \(_1\) before and after bronchodilator use. Furthermore, we showed that COPD with FEV \(_1\) reversibility was not associated with atopy or asthma. In fact, our findings corroborate those of previous studies showing that, although IL-6, IL-8 and TNF levels are higher in COPD patients than in healthy subjects, the production of those cytokines is comparable between the two types of COPD.

Historically, the incidence of COPD has been highest among males and among smokers. However, the proportional representation of women has been increasing. ([18]) In addition, a marked female predominance has been reported among patients with an early onset of severe COPD. ([19]) In the present study, the proportional distribution of males and females was similar between the two patient groups, as was the proportion of smokers.

The analysis of sputum is an useful tool in the evaluation of inflammation of the airways. ([15]) Induced sputum was initially used for the diagnosis of lung cancer and later for infectious diseases. In the early 1990s, this method was employed in the investigation of bronchial inflammation associated with asthma. ([20]) More recently, because of its safety, reproducibility and low cost, it has been used for investigation of the pathogenesis of asthma and COPD. Local neutrophil recruitment in inflammation is a hallmark of COPD, as is an increase in the levels of inflammatory mediators in the airways and in circulating blood. ([21]) The release of neutrophil elastase, acid phosphatase, and myeloperoxidase that occurs during neutrophilic inflammation is characteristic of COPD. ([22-24]) Neutrophils are the predominant cells in the sputum of COPD patients, and high proportions of neutrophils were found in the sputum of the patients in both of the COPD groups evaluated in the present study. ([3]) However, it is also possible that the hypertonic...
saline solution used for the induced sputum techniques contributes to increasing the number of neutrophils.\(^{(25)}\)

The role that eosinophils in the sputum play in COPD is not clear. It was once thought that the presence of eosinophils was related to a subgroup of COPD patients with features of asthma\(^{(2,5)}\) and that it was associated with a better response to corticosteroid therapy.\(^{(26)}\) In fact, an association between asthma and COPD would further the development of strategies for the therapeutic management of COPD.\(^{(27,28)}\) However, our data argue against the occurrence of asthma in patients who show post-bronchodilator FEV\(_1\) reversibility. None of our patients had a personal or family history of asthma, and the prevalence of atopy, as determined with the skin prick test, was comparable between the two patient groups. In addition, the immunological profile (cytokine and chemokine production) was similar in the two groups, and there was no increase in RANTES (CCL5), which is a typical Th2 cytokine in COPD patients with post-bronchodilator FEV\(_1\) reversibility. In a previous study, we found an association between nasal eosinophils and atopy in patients with COPD.\(^{(29)}\) However, in the present study, the presence of eosinophils in the sputum was not found to be associated with atopy or asthma. Although the role that eosinophils play in COPD is not completely understood, we observed that eosinophil counts were increased in COPD patients with post-bronchodilator FEV\(_1\) reversibility. We also identified an inverse correlation between the proportion of eosinophils in the sputum and the decrease in FEV\(_1\). Although our findings might suggest that eosinophils are related to the severity of COPD, no eosinophils were found in the sputum of the patients with stage IV COPD. It is possible that, as in the later stages of the disease, the numbers of inflammatory cells are decreased in that phase of the disease.

Increased levels of IL-6, IL-1\(\beta\), TNF, and IL-8 have been observed in the induced sputum of patients with stable COPD.\(^{(5)}\) There is also evidence of a relationship between elevated cytokine levels in COPD and cigarette smoking.\(^{(6,30)}\) However, the relationship that chemokines have with eosinophils in the sputum or with FEV\(_1\) reversibility has not been evaluated. Serum IL-6 is considered the best biomarker of COPD severity when associated with the degree of airway obstruction and has been associated with mortality.\(^{(7)}\) We found that the severity of COPD did not correlate with the serum levels of IL-6, IL-8, TNF, or RANTES (CCL5). In the

Figure 2. Dot plots of serum levels of the cytokines TNF (A), IL-6 (B), and IL-8 (C), as well as those of RANTES (CCL5; D), in healthy controls (HC), COPD patients with non-reversible airflow limitation (non-RAL), and COPD patients with partially reversible airflow limitation (partial-RAL). *p < 0.05, †p < 0.01, and ‡p < 0.001; Kruskal-Wallis test followed by Dunn’s post-test for multiple comparisons.
Eosinophilic airway inflammation has been associated with COPD exacerbations. A reduction in sputum eosinophil counts has been associated with a reduction in COPD exacerbations. Because this was a cross-sectional study, we did not assess the relationship between the inflammatory response and exacerbation. However, we found an association between eosinophil inflammation and airway obstruction. That supports the relationship between eosinophilic airway inflammation and COPD exacerbation, as well as the association between the peripheral blood eosinophil count and death from exacerbations of COPD.

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