Marked Up-regulation of T Lymphocytes and Expression of Interleukin-9 in Bronchial Biopsies From Patients With Chronic Bronchitis With Obstruction*

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Study objective: To examine the differences in the inflammatory cell and cytokine profile between patients with chronic bronchitis (CB) with and without airway obstruction compared to control subjects.

Design: We used bronchial biopsy samples from the patients and control subjects and analyzed them for the presence of CD3 T cells, CD68, major basic protein (MBP), elastase, and tryptase, as well as expression of messenger RNA (mRNA) coding for interleukin (IL)-4, IL-5, interferon (IFN)-γ, IL-9, eotaxin, and IFN-γ-inducible protein (IP)-10. The techniques of immunocytochemistry and in situ hybridization were used. Results were expressed as the number of immunoreactive and mRNA-positive cells per field.

Results: Increased number of elastase, CD68, and MBP-positive cells (n = 9, p < 0.01) was demonstrated in both groups of patients with CB compared to control subjects. In patients with CB and obstruction, the number of elastase, CD68, and the number of CD3-positive cells was significantly increased compared to patients with CB without obstruction (n = 9, p < 0.01). IFN-γ mRNA expression was increased in both groups of patients with CB compared to control subjects (n = 9, p < 0.01). IL-9 mRNA was significantly increased only in patients with CB and obstruction (n = 9, p < 0.01). Co-localization studies demonstrated > 80% of all IL-9–positive cells to be CD3-positive T cells. IP-10 mRNA was significantly increased in both groups of patients with CB compared to control subjects (n = 9, p < 0.01).

Conclusions: These results indicate a differential expression of inflammatory markers and cytokine mRNA in patients with obstructive CB. Increased presence of T lymphocytes and up-regulation of IL-9 and IP-10 mRNA expression in the bronchial biopsy samples may contribute to the airway obstruction in these patients.

Key words: bronchial biopsies; chronic bronchitis; COPD; cytokines; immunocytochemistry; inflammation; interleukin-9

Abbreviations: CB = chronic bronchitis; IFN = interferon; IL = interleukin; IP = interferon-γ-inducible protein; MBP = major basic protein; mRNA = messenger RNA; Th1 = T-helper type 1; Th2 = T-helper type 2

COPD is a multifactorial disease of the airways characterized by the presence of irreversible airway obstruction due to chronic bronchitis (CB) or emphysema, protease-antiprotease imbalance, oxidative stress, and recurrent infections. Evidence has suggested that chronic airway inflammation and structural remodeling are integral components in the development of chronic airflow obstruction seen in COPD.1-5 One of the prominent features of COPD histology is epithelial and subepithelial influx of inflammatory cells5,6,7 and increased expression of many enzymes.8,9 These inflammatory changes may predispose the individual to development of centrilobular emphysema and cause destruction of alveolar attachments resulting in reduced lung function, that is, a fall in FEV1. Many of these features of COPD are believed to be caused by damage to the airways by neutrophil and eosinophil mediators, perforin and granzymes. Chronic mucosal airway inflammation in COPD is regulated by a highly complex network of regulatory mechanisms.

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mutually interacting cytokines. Within this network, the exact contribution of each cytokine remains to be fully established.

Unlike asthma, we know little about the cells that initiate and drive the inflammatory process in COPD. It has previously been shown that CD8+ T cells are overexpressed in the lungs of patients with COPD, and that these cells are inversely related to lung function.\(^{10}\) COPD is frequently considered to be a T-helper type 1 (Th1)-driven disease associated with lymphomononuclear infiltration into the mucosa and presence of neutrophilic inflammation. Reports have documented recruitment of eosinophils and recovery of significant amounts of eosinophilic cationic protein in BAL fluid during exacerbations of chronic bronchitis,\(^{11}\) as well as increased interleukin (IL)-4 and IL-5 gene expression in the mucus-secreting glands of smokers with bronchitis.\(^{12}\) In the later study,\(^{13}\) there was no significant association between the numbers of IL-4 and IL-5 messenger RNA (mRNA)-positive cells and the number of CD8+ cells. The role of cytokines in CB and their contribution to airway obstruction in these patients remain to be fully explored.

To examine the differences in the inflammatory cell and cytokine profile in patients with CB with and without airway obstruction, we used bronchial biopsy samples from these subjects and compared them to healthy control subjects. The specimens were analyzed for the presence of inflammatory cells including T lymphocytes (CD3), macrophages (CD68), eosinophils (major basic protein [MBP]), and neutrophils (elastase), as well as the expression of mRNA coding for cytokines IL-4, IL-5, IL-9, interferon (IFN)-γ, eotaxin, and IFN-γ–inducible protein (IP)-10.

**Materials and Methods**

**Subjects**

Nine patients fulfilling the diagnostic criteria for CB with documented airways obstruction and nine patients with CB without obstruction were recruited from the Department of Pneumoinmunology, Calmette Hospital in Lille, France. Diagnosis was defined according to the American Thoracic Society criteria.\(^{13}\) Briefly, the patients received a diagnosis of obstructive CB if they had a history of cough and sputum production on most of the days of the month for at least 3 months a year during the 2 years before the study. Airway limitation was defined as an FEV\(_1\) < 80% of predicted with a < 15% improvement in FEV\(_1\) after inhalation of 400 μg of salbutamol. All patients with CB had a history of cigarette smoking. Subjects with lung disease other than CB or with systemic disease affecting the lungs were excluded from the study.

The results were compared with age- and sex-matched healthy, nonsmoking control subjects (n = 9) recruited from the Montreal Chest Hospital in Montreal, Canada. None of the patients had received either oral or inhaled corticosteroids or had any exacerbations during the 12 weeks preceding the study. None of the subjects had a history of respiratory tract infection within 6 weeks preceding the study. All subjects were nonatopic as determined by skin-prick testing and had no clinical history of allergic disease. This study was approved by the Montreal Chest Institute and the Calmette Hospital Ethics Committees in France. Written informed consent was obtained from all patients before undergoing fiberoptic bronchoscopy. Clinical characteristics of all patients enrolled in this study are shown in Table 1.

**Tissue Preparation**

Bronchial biopsy specimens were obtained from the subsegmental airways using the technique of fiberoptic bronchoscopy. Biopsy samples for in situ hybridization were immediately fixed in 4% paraformaldehyde for 2 h, washed in 15% phosphate-buffered saline-sucrose solution, and blocked in optimal cutting temperature embedding medium (Tissue-Tek; Somagen Diagnostics; Edmonton, CA) by snap-freezing in isopentane cooled in liquid nitrogen. Tissue was sectioned (5 μm) using a cryostat (Microm HM 500; Carl Zeiss; Montreal, Canada), placed onto poly-L-lysine–coated glass slides, baked overnight at 37°C and stored at −80°C until further use. For immunocytochemistry, biopsy samples were not fixed but placed in ice-cold phosphate-buffered saline solution, fixed in acetone-methanol, and stored at −20°C.

**Immunocytochemistry**

To ascertain the phenotype of inflammatory cells present in the biopsy samples, immunohistochemical markers for T lymphocytes (CD3; DAKO Diagnostics; Mississauga, ON, Canada), macrophages (CD68; DAKO Diagnostics), eosinophils (MBP; DAKO Diagnostics) and neutrophils (elastase; DAKO Diagnostics) were used. Immunocytochemistry was performed by using a modified alkaline phosphatase antialkaline phosphatase method as previously described.\(^{14}\) In some slides, double immunocytochemistry was performed by co-incubating the tissue overnight with both CD3 monoclonal antibody and IL-9 goat antihuman primary antibodies. Briefly, tissue was blocked (universal blocking solution; DAKO Diagnostics) for 15 min and incubated with optimum concentration of the primary antibody overnight at 4°C in a humidified chamber. The next day, slides were washed in Tris-buffered saline solution (pH 7.2), incubated with the sec-
in situ hybridization. For negative controls, primary antibody was replaced by an isotype-matched control antibody.

Cytokine mRNA expression for IL-4, IL-5, IL-9, IFN-γ, and IP-10 in bronchial biopsies was determined using radiolabeled complementary riboprobes (complementary RNA antisense). The complementary DNA sequences for these cytokines were inserted into vectors (Promega pGEM; Fisher Scientific; Nepean, ON, Canada), grown in Escherichia coli, and linearized in vitro transcription to generate sense and antisense probes was performed in the presence of 35S-uridine triphosphate and the appropriate T7 or SP6 polymerases as previously reported.15,16 Bronchial biopsies were permeabilized, treated with acetic anhydride and triethanolamine to reduce the nonspecific binding, and prehybridized in formamide at 42 °C. 35S-labeled antisense probes were used (106 counts per minute per section), followed by high-stringency posthybridization washings. Unhybridized single-stranded RNA was removed by treating the preparations with a solution containing ribonuclease. After posthybridization, slides were immersed in emulsion fluid and exposed for 14 days. Slides were counterstained with Gill II hematoxylin, and a positive signal was identified as silver grains over the cells.

Data Analysis

In the airway epithelium and submucosa, the number of positive cells was averaged from six to eight random, nonoverlapping fields, and expressed as the mean number of positive cells per field ± SD. Cell counts were performed blindly by two independent observers using an Olympus light microscope (Carson Group; Markam, ON, Canada) [< 400 × magnification with an eyepiece graticule of 0.202 mm2]. The level of agreement between blinded counters was > 90%. Significant differences between groups of patients were detected by Student t test, and significance was accepted at the level of 95%

RESULTS

Inflammation in Bronchial Biopsy Samples of Patients With CB

In both groups of patients with CB, neutrophils (elastase-positive cells) and T lymphocytes (CD3-positive cells) comprised the majority of inflammatory cells in the bronchial biopsy samples (Fig 1). In patients with CB without obstruction, there was a significant increase in the number of elastase (4.4-fold), CD68 (2.3-fold), and MBP (11-fold)-positive cells compared to healthy control subjects (n = 9, p < 0.01) but no increase in CD3-positive T cells (Fig 1). In patients with CB and obstruction, there was a significant increase in the number of elastase (7.6-fold), CD68 (3.4-fold), and MBP (sevenfold)-positive cells compared to healthy control subjects (Fig 1). The increase in elastase-positive cells in patients with CB and obstruction was significantly higher than the increase seen in patients with CB without obstruction (n = 9, p < 0.01) [Fig 1]. No difference was observed in the number of CD3-positive cells or the number of MBP-positive cells between the two groups of patients with CB (with or without obstruction) [Fig 1].

Cytokine Expression in Bronchial Biopsy Samples of Patients With CB

Variable level of constitutive expression of IL-4 mRNA, IL-5 mRNA, IL-9 mRNA, and IFN-γ mRNA was detected in all patients (Fig 2). There was no difference in the number of IL-4 mRNA or IL-5 mRNA-positive cells in patients with CB compared to control subjects or patients with CB with or without obstruction (Fig 2). The most frequently expressed cytokine mRNA in patients with CB was IL-9 mRNA followed by IFN-γ mRNA (Fig 2). Patients with CB and obstruction had significantly increased number of IL-9 mRNA-positive cells compared to patients with CB without obstruction (4.9-fold increase) [n = 9, p < 0.01] and compared to control subjects (sixfold increase) [n = 9, p < 0.01; Fig 2]. Double immunocytochemistry demonstrated that in patients with CB and obstruction, > 80% of all the CD3-positive T cells in this tissue are IL-9 positive. The expression of IL-9 mRNA-positive cells in patients with CB without obstruction was similar to that in control subjects. Both groups of patients with CB may have increased number of IFN-γ mRNA-positive cells compared to control subjects (n = 9, p < 0.01); however, no difference

Table 1—Clinical Characteristics of the Patients*

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>No.</th>
<th>Age, yr</th>
<th>Male/Female Gender</th>
<th>Smoking Status, Pack/yr</th>
<th>FEV1, % Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>46 ± 9.7</td>
<td>6/3</td>
<td>Nonsmokers</td>
<td>98.0 ± 10.0</td>
</tr>
<tr>
<td>CB without obstruction</td>
<td>9</td>
<td>48 ± 12.1</td>
<td>7/2</td>
<td>26.3 ± 12.8</td>
<td>98.5 ± 15.4</td>
</tr>
<tr>
<td>CB with obstruction</td>
<td>9</td>
<td>57.3 ± 11.1</td>
<td>9/0</td>
<td>69.0 ± 37.6</td>
<td>55.8 ± 11.5</td>
</tr>
</tbody>
</table>

*Data are represented as mean ± SD unless otherwise indicated.
was observed between patients with CB with or without obstruction (Fig 2).

**Chemokine Expression in Bronchial Biopsy Samples of Patients With CB**

Constitutive expression of eotaxin mRNA and IP-10 mRNA was detected in all patients (Fig 3).

Expression of eotaxin mRNA was significantly reduced in patients with CB without obstruction (2.3-fold) compared to control subjects (n = 9), \( p < 0.01 \) vs healthy control subjects (white bars, n = 9); \( \dagger\dagger p < 0.01 \) vs patients with CB without obstruction.

There was no difference in the number of eotaxin mRNA-positive cells between the two groups of patients with CB and obstruction (black bars, n = 9) and in patients with CB without obstruction (gray bars, n = 9).

**FIGURE 1.** Immunodetection of CD3, elastase, CD68, and MBP-positive cells in patients with CB and obstruction (black bars, n = 9) and in patients with CB without obstruction (gray bars, n = 9). Results are expressed as mean number of positive cells per millimeter squared ± SD. **p < 0.01 vs healthy control subjects (white bars, n = 9); \( \dagger\dagger p < 0.01 \) vs patients with CB without obstruction.

**FIGURE 2.** Expression of IL-4 mRNA, IL-5 mRNA, IL-9 mRNA, and IFN-γ mRNA in patients with CB and obstruction (black bars, n = 9) and in patients with CB without obstruction (gray bars, n = 9). Results are expressed as mean number of positive cells per millimeter squared ± SD. **p < 0.01 vs healthy control subjects (white bars, n = 9); \( \dagger\dagger p < 0.01 \) vs patients with CB without obstruction.
patients with CB. IP-10 mRNA was significantly increased in both groups of patients with CB compared with control subjects (threefold) [n = 9, p < 0.01], and no difference in IP-10 mRNA expression was observed between patients with CB with or without obstruction (Fig 3).

**DISCUSSION**

In this study, we have characterized the inflammatory profile and the expression of mRNA for both Th1 and T-helper type 2 (Th2) cytokines and chemokines in bronchial biopsy samples from patients with CB without obstruction and patients with CB and obstruction (COPD), and have compared these results to healthy control subjects. We have shown neutrophil, macrophage, and T-lymphocyte inflammation in bronchial mucosa of patients with CB with or without obstruction, and our results are in agreement with previous findings from BAL samples and mucus-secreting glands of patients with COPD.17–20 Furthermore, we have shown increased number of eosinophils and increased expression of IFN-γ mRNA and IP-10 mRNA in these patients. In addition, for the first time we have demonstrated that the bronchial inflammation in patients with CB and obstruction is more severe compared to patients with CB without obstruction, and this inflammation is characterized by infiltration of T lymphocytes and a dramatic increase in the expression of IL-9 mRNA.

IL-4 and IL-5 are typical Th2 cytokines that play a pivotal role in the pathogenesis of asthma; however, their role in CB is largely unknown. There are contradictory reports in the literature showing both increased number of IL-5–positive lymphocytes in sputum of patients with CB,21 or no change in the expression of IL-5 protein in bronchial mucosa in patients with CB during exacerbations.22 In our study, we have shown a similar degree of IL-5 mRNA (and IL-4 mRNA) expression in patients with CB and healthy control subjects; however, patients with CB had significantly higher numbers of MBP-positive eosinophils in their bronchial biopsy samples. This finding is in agreement with previous reports22,23 that demonstrated marked eosinophilia in bronchial mucosa of patients with COPD; however, unlike in asthma, they failed to show an increase in the number of IL-5–positive cells compared to healthy control subjects. Although IL-5 is a major cytokine involved in differentiation, maturation, and recruitment of eosinophils into the inflammatory sites, our results strongly suggest that IL-5 (or IL-4) alone is unlikely to be responsible for orchestrating eosinophil recruitment into the bronchial mucosa of patients with CB, or to play a crucial role in the pathogenesis of this disease.

IL-9 is a Th2 cytokine that has pleiotropic effects on many cell types, in particular T lymphocytes, eosinophils, neutrophils, and mast cells. It up-regulates the expression of the high-affinity IgE receptor (FcRI) in mast cells24 and stimulates their production of Th2 cytokines.25 IL-9 also promotes proliferation of CD8+ T cells26 and selective over-expression of IL-9 in transgenic mice results in in vivo infiltration of T cells into the airways.27 More recently, our laboratory has demonstrated the ability of IL-9 to up-regulate mucus production in asthmatic patients through induction of hCLCA1 gene expression.28 In this study, we have demonstrated a large increase in the number of IL-9 mRNA-positive cells only in the bronchial mucosa of patients with CB and obstruction and not patients with CB without obstruction or healthy control subjects (Fig 2). These results support the idea that this large and selective increase in the expression of IL-9 mRNA may stimulate production of mucus and may be the direct cause of airway obstruction seen in these patients. Although IL-9 is produced by mast cells, eosinophils, and neutrophils, the major source of IL-9 in asthmatics has been shown to be T lymphocytes (in particular CD4+ T cells). In this study, we have shown for the first time that in patients with CB and obstruction, T lymphocytes are also an important source of IL-9. Previous results in our laboratory using this same cohort of patients with CB have demonstrated that the majority of these T cells are CD8+ T cells (unpublished observations). The evi-
idence in support of T cells production of IL-9 in our patients is twofold. Firstly, we have shown an increased number of CD3-positive cells to be associated with increased IL-9 protein immunoreactivity only in patients with CB and obstruction. Secondly, we have demonstrated a majority of CD3-positive cells in the tissue also to be positive for IL-9. Double immunocytochemistry has further shown that epithelial cells are an additional source of IL-9.

The only other cytokine whose expression was elevated in diseased biopsy specimens compared to healthy control subjects was IFN-γ. Although statistically significant, this increase was small compared to increases in IL-9 mRNA seen in the same tissue (Fig 2). Similar low levels of IFN-γ and IFN-γ-producing cells have previously been reported in airway tissue29 and in peripheral blood30 of patients with COPD. No difference was detected in the expression of IFN-γ mRNA between the two groups of patients with CB, suggesting an unlikely role for IFN-γ as being an important player in the cause of obstruction in patients with CB.

As mentioned, in this study we have shown increased number of MBP-positive cells in bronchial biopsies of patients with CB (Fig 1). Eosinophilia has previously been thought to be present mainly in exacerbations of COPD.1,31 Since we found eosinophilia not to be associated with IL-5 mRNA expression, we studied the immunoreactivity of eotaxin in these biopsy samples. Eotaxin is an eosinophil-specific chemokine. Although the role of eotaxin in asthma has been extensively studied, there are only a few studies examining the role of eotaxin in the pathogenesis COPD and/or CB. Surprisingly, it has been previously reported that the eotaxin levels are higher in serum of patients with COPD than the levels found in asthmatics.32 Expression of eotaxin mRNA in COPD has been localized to lymphomononuclear cells, the endothelium, and airway epithelium33; however, we failed to see an increase in the expression of this chemokine in patients with CB when compared to control subjects (Fig 3), despite marked eosinophil infiltration seen in these patients. These findings suggest that eotaxin is not involved in recruitment of eosinophils in patients with CB. Furthermore, these results are consistent with previous findings by Miotto and colleagues,34 who showed in bronchial biopsies that eotaxin was increased only in asthmatics and not in patients with COPD, concluding that eotaxin expression is specifically associated with lung diseases of a Th2 cytokine profile.

To date, much research has focused on the role of IL-8 and monocyte chemotactic proteins in diseases of chronic inflammation.35 Our data suggest that another chemokine, IP-10, may play a role in COPD and/or CB inflammation. IP-10 is involved in recruitment of neutrophils and activated lymphocytes, hence have originally been associated with Th1-mediated diseases. We have found significantly increased expression of IP-10 mRNA in both groups of patients with CB compared to control subjects (Fig 3), and this chemokine may be responsible for mediating recruitment of not only CD68, CD3, and elastase-positive cells into the bronchial mucosa, but also eosinophils. Jinguan and colleagues36 previously demonstrated the presence of IP-10 receptor, CXC chemokine receptor 3 (CXCR3), to be expressed on eosinophils. In our patients with CB, the increase in IP-10 was associated with increase number of eosinophils in the tissue (Fig 1), as well as increased expression of IL-9 mRNA (Th2 cytokine) compared to control subjects.

In summary, we have demonstrated that inflammation in patients with CB without obstruction is clearly characterized by increased presence of not only neutrophils and macrophages as originally proposed but also increased eosinophilia. The inflammation in patients with CB and obstruction was more severe and characterized by abundance of T lymphocytes, which was associated with a significant increase in the RNA expression of both Th1 (IFN-γ) and Th2 (IL-9) cytokines. The up-regulation in IL-9 expression may be directly involved in mucus hypersecretion, airway smooth-muscle hyperplasia, and remodeling, leading to obstruction and airway hyperresponsiveness, which are typical features in this disease. While obvious pathologic difference have previously been documented between patient with COPD and asthmatics,19,29,37,38 this is the first evidence showing that COPD and CB is driven by not only Th1- but Th2-immune response, and hence may exhibit greater similarities to asthmatics than originally thought.

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