Different cytokine patterns in bronchial biopsies in asthma and chronic bronchitis

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Bronchial biopsies have made possible the detailed study of the pathology of the airways of humans with respiratory disease. Much data has been accumulated on asthmatics or normal controls but much less is known about chronic bronchitics. The aim of this study was to characterize the cellular and cytokine pattern seen in chronic bronchitics and to compare these with control and asthmatic subjects. The patients were also characterized clinically. In this study, immunocytochemistry on cryostat sections from bronchial biopsies were used to determine the level of inflammatory cells and cells of the immune system as well as the pattern of cytokines. This study revealed a distinct cellular and cytokine pattern for each of the three different patient groups, although the diversity of the cytokines analysed was limited by the size of the biopsies. In the inflammatory infiltrate of patients with asthma, CD4+ T-cells and eosinophils were the most prominent cell types discerned. All of the expected cytokines such as IL-1, TNF-a, IL-4, IL-5 and IFN-γ were found. In contrast, the emphasis in chronic bronchitic patients was quite different. The predominant cell types were macrophages, neutrophils, mast cells and CD8+ T-cells, but eosinophils were also abundant. In addition, IL-4 and TNF-a were the only cytokines present of those tested.

Introduction

Asthma and chronic bronchitis (CB) are recognized as two diseases of the conducting airways which contribute significantly to morbidity and mortality world-wide (1). Until the advent of fibre-optic bronchoscopy, little was known about the mechanisms underlying asthma. Biopsy studies comparing the histology of patients with asthma with normal control subjects have demonstrated that an airway inflammatory process is present even in mild asthmatics (2-4) or in patients with newly-diagnosed asthma (5). In these studies, elevated numbers of eosinophils, activated lymphocytes and macrophages were constant features observed in asthmatics as compared with normal controls (3,4,6-8). The numbers of mast cells or neutrophils, however, did not show significant changes in mild to moderate asthmatics as compared with normal subjects (7,9,10).

Bronchial inflammation may be due in part to the release of cytokines by activated cells. Recent studies using in situ hybridization on bronchoalveolar lavage (BAL) cells and biopsies suggested that a TH2-like cytokine pattern existed in asthma, with CD4+ T-cells being the major cytokine-producing cell type (11,12). In addition, an increase in the number of IL-5 mRNA positive cells could also be demonstrated in eosinophils from asthmatics (13), and mast cells have also been found to release cytokines in asthmatic airways (14).

Inflammation is also known to be a feature of CB (15,16). Neutrophils, macrophages and T-cells (17-19), but also eosinophils (20-22) have been found in the airways of patients with CB. The cytokine expression pattern in these CB patients has not yet been established in detail although TNF-a was found to be increased mainly during exacerbations (22). Judging by the assumed cause of this disease, it would be expected that a cytokine profile characteristic of chronic inflammation would be predominant,
Table 1  Demographic characteristics of the patients

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<tr>
<th>Patient No.</th>
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<th>FEV₁</th>
<th>Smoking†</th>
<th>Treatment‡</th>
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*Aas, Asthma symptom score [grades of asthma on a scale from 1 (mild) to 5 (severe)] (35).
†Stop after x yr of smoking. ‡β, β-agonist. §cigarettes day⁻¹. n.d., not determined.

e.g. IL-1, IFN-γ, IL-2 and TNF-α, but not IL-4 or IL-5. Again the eosinophil is present in large numbers but, in contrast to the asthmatic tissue, there is little suggestion of degranulation (20).

In spite of their common inflammatory component, no direct comparison between asthma and CB in terms of cell and cytokine pattern has been carried out so far. The aim of this study was to characterize the most important cell types and cytokines in bronchial biopsies derived from patients with these diseases and to compare them with healthy control subjects. By careful comparison with the clinical data, it was hoped that some insight might be gained which would contribute to the design of new specific drugs. In order to demonstrate the presence or absence of cells relevant to inflammation, immunocytochemistry was carried out on cryostat sections with markers for macrophages, mast cells, T-cell subsets, eosinophils, neutrophils and the cytokines IL-1, IFN-γ, TNF-α, IL-4 and IL-5. GM-CSF was not studied as it has recently been shown that GM-CSF immunoreactivity was similar in CB and control biopsies (Vignola, unpubl. data) and also at a low level by comparison with asthma (23).

Methods

Subjects

Eleven patients with a well-documented history of asthma, six patients with CB and five healthy control subjects were chosen for this study. Their clinical characteristics are summarized in Table 1. The type of disease and status were carefully defined as previously described (20).

Fibre-optic Bronchoscopy

Fibre-optic bronchoscopy was carried out as previously described (4). The biopsy was removed under direct vision using alligator forceps in subsegmental bronchi of the left lower lobe. After excision of the tissue, the biopsy was frozen immediately and stored at −80°C until transported to Basel, Switzerland on dry ice. The study was approved by the Ethics Committee of the hospital and subjects gave their informed consent.

Antibodies

Murine monoclonal antibodies were purchased from Dakopatts (Glostrup, DK): CD68-DAKO-
Macrophage (M718) reacts with human macrophages. DAKO-CD4 (MT 310) and -CD8 (DK 25) are specific for helper/inducer and suppressor/cytotoxic T-cells, respectively. DAKO-Elastase (NP 57) is specific for human neutrophil elastase. Eosinophils were demonstrated with the mAb EGI1 to human eosinophilic cationic protein (ECP, Kabi Pharmacia, Duebendorf, Switzerland). This antibody recognizes both the stored and secreted forms of ECP (24). Mast cells were demonstrated with the mAb AA1 raised against human mast cell tryptase (25). The mAb 2D8 was used for the detection of IL-1β (26). Its specificity was assessed using lipopolysaccharide (LPS) stimulated monocytes. For the detection of IL-4, the three mAbs 8F12, 4D9 and 3H4 raised against different epitopes of murine IL-4 were used as a cocktail. Their specificity was demonstrated on Chinese hamster ovary cells transfected with IL-4 (27). IFN-γ was detected with the mAb 42-103 (28). The presence of IL-5 was revealed using the mAb 7 which is specific for IL-5 (29). The anti-TNF-α polyclonal antibody was purchased from Genzyme (Cambridge, MA, U.S.A.). The rabbit anti-mouse antibody and the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex were obtained from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

**IMMUNOLABELLING**

The biopsies were mounted in Tissue Tek 5 Miles (Elkhart, IN, U.S.A.) and 8 μm cryosections were cut on a cryostat at −22°C. Sections were picked up on a microscope slide, air-dried, wrapped in aluminium foil and stored at −80°C until use. They were allowed to defrost before they were unwrapped for immunostaining. All steps were carried out at room temperature. Depending on the antigen, sections were fixed with either Tris-buffered saline (TBS: 50 mM Tris/HCl, pH 7.4, 0.15 NaCl) containing 2% formaldehyde (Polyscience, Warrington, PA, U.S.A.) for 20 min, or in 100% acetone (Fluka, Buchs, Switzerland) for 10 min. After fixation, the slides were washed in TBS and non-specific binding sites and free Fcy receptors were blocked with TBS containing 0.05% NP-40 (Pierce, Rockford, IL, U.S.A.), 1% bovine serum albumin (BSA: Sigma, St Louis, MO, U.S.A.), 5% normal rabbit serum (NRS: SeraLab, Sussex, U.K.) and 50 μg ml⁻¹ human IgG (Jackson Immunoresearch) for 30 min. The primary antibodies were diluted in incubation buffer (TBS containing 0.05% NP-40, 1% BSA and 1% NRS) and applied to the section for 1 h. Sections were then washed with TBS/0.05% Tween 20 (Pierce) for 3 x 10 min. Monoclonal antibody binding was detected by a modification of the APAAP method of Mason (30). Rabbit anti-mouse antibody diluted 1/50 in incubation buffer was applied for 30 min and the sections washed as above. The sections were then covered with the APAAP complex diluted 1/50 in incubation buffer, incubated for 30 min and then washed as above. The rabbit anti-mouse and the APAAP steps were repeated once to increase signal intensity. Sections were finally washed in Tris/HCl pH 8.2 and the alkaline phosphatase was developed using Fast Red as the chromogen, yielding a red precipitate (30). Levamisole (Sigma) was included in the colour reaction at a final concentration of 5 mm in order to block endogenous alkaline phosphatase (31). Sections were counterstained with Harris' haematoxylin (Merck, Dietikon, Switzerland) for 5 s. Cell preparations were mounted in 90% glycerol/10% H₂O. Control slides were treated in the same way but omitting the primary antibody.

Quantification of staining and analysis of results: Sections were examined using a Zeiss Axioskop microscope (Oberkochen, Germany). For patients with asthma, one section was examined for each antibody due to the very small size of the samples available. For patients with CB and healthy control subjects, at least two sections were analysed for each antibody. All the ratings for the staining was performed on coded sections by one investigator (GB) who was not aware of the status of the patient. Staining was assessed semi-quantitatively as follows: 0, no; 1, weak staining; 2, moderate staining; 3, strong staining. This assessment of staining is closer to being logarithmic than linear. Very small sections could not be rated satisfactorily and had to be excluded. The accuracy of this quantification method had been verified in the mouse lung where detailed counts of positive cells per unit area were made using image analysis, and a direct comparison with the semi-quantitative method was made.

**STATISTICAL ANALYSIS AND EXPRESSION OF THE DATA**

Final data was expressed as means ± SEM for patients with asthma, CB and healthy control subjects. The Fischer exact test was used for comparisons.

**Results**

This biopsy study was carried out with a total of 22 subjects: 11 asthmatics, six chronic bronchitics and five healthy control subjects. A mucosal inflammatory infiltrate was observed in all subjects studied. This infiltrate often demonstrated a pronounced concentration of cells beneath the epithelial basement membrane but was otherwise equally...
distributed throughout the whole tissue sample. A summary of all the biopsy finding can be seen in Figs 1 (cell types identified), 2 (positive cytokine signal) and 3 (percentage of patients with a positive signal).

From Fig. 1, it can be seen that both asthmatics and patients with CB showed an increase in the number of macrophages, eosinophils and both CD4+ and CD8+ T-lymphocytes as compared with normal control subjects. Patients with CB showed an increase in the number of mast cells as compared with both asthmatic and normal control subjects, while the number of neutrophils was decreased in asthmatics as compared with patients with CB and normal control subjects.

The presence of all five cytokines investigated (Fig. 2) could be detected in asthmatic patients, with IL-4 and IFN-γ being predominant. In patients with CB, only IL-4 and TNF-α could be detected on occasion, and in normal control subjects both IL-4 and IL-5 could be found but only rarely.

The data was analysed using the Anova statistical system [Fischer PLSD (exact) test]. The small numbers of samples prevented definitive statements being made but distinct trends were noted. Table 2 gives the results where a significant difference was seen, i.e. the increase in macrophages over control for both asthma and CB, and the increase in IL-4 positive cells in CB as compared with control.

Figure 3 gives the incidence of the cytokines and Fig. 4 compares the individual scoring for cytokines and cell types found present in the different patient groups. As always in these studies, a big variation within groups was observed. In all the sections, the presence of macrophages could be detected [Fig. 4(a)]. Patients with CB showed a consistent increase in the number of macrophages as compared with healthy control subjects. In six of 11 patients with...
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IFN-γ, IL-4, IL-5, TNF-α, IL-1β

Fig. 3 Percentage of patients with asthma (solid bar), chronic bronchitis (stippled bar) and normal controls (open bar) in which a positive signal for the individual cytokines could be detected.

In asthma, the number of macrophages was increased when compared with normal subjects. IL-1β staining was seen in patients with asthma (3/11) but in neither of the other two study groups, despite the presence of large numbers of macrophages. TNF-α was detectable in both patients with asthma (2/7) and CB (1/6). Control subjects showed no staining for this cytokine.

The number of mast cells as seen by the presence of mast cell specific tryptase showed big variations between the different groups [Fig. 4(b)]. Overall, the average number was increased in patients with CB as compared with asthmatics and normal control subjects. Mast cells in biopsies from asthmatics often had a degranulated appearance. The presence of IL-4 was a consistent observation in subjects with asthma. Eight of 11 patients were positive for IL-4. These positive cells were mainly located close to the basement membrane. In patients with CB, one of six patients was IL-4 positive, and in healthy controls, one of five patients was IL-4 positive.

Similar numbers of CD4+ T-lymphocytes were seen both in patients with asthma and CB. The average numbers in these two groups were elevated as compared with control subjects. In patients with CB, a clear increase in the ratio of CD8+/CD4+ T-cells could be seen. IFN-γ expression was confined to subjects with asthma, where four of nine patients were positive. This cytokine could not be detected in the other two groups (Fig. 3).

Eosinophils were not seen in control subjects [Fig. 4(c)] although two of three normal controls were weakly positive for IL-5 suggesting the involvement of a different cell type. Both patients with asthma and CB showed considerable numbers of eosinophils. Three of nine asthmatic patients were positive for IL-5 while no IL-5 could be seen in CB patients.

Patients with CB showed an increase in the number of neutrophils as compared with patients with asthma. Two of four normal controls showed high numbers of neutrophils as well.
Discussion

As with any clinical study, the importance of careful characterization of the patient cannot be over-emphasized. Guidelines drawn up by the American Thoracic Society (32) were strictly adhered to and the parameters used have been established and thoroughly tested in previous studies (4,20). As can also be seen in Table 1, the clinical data for both patients and controls have been recorded and correlated with the findings from the biopsy samples.

Obviously with bronchial biopsies, the amount of tissue obtained is very small, only allowing a limited number of sections to be cut from each one. In turn, this affects the choice of cytokine and cell markers that can be used. The cytokines chosen for study in the asthmatic patients were those already well established as being upregulated in asthma, with the exception of GM-CSF as mentioned earlier (23), as this part of the study was designed to confirm or extend data already obtained. The markers could be divided into two groups, those related to the immunological component e.g. T-cells, and those related to the inflammatory component e.g. macrophages or neutrophils.

When investigating CB, the relevance of taking bronchial biopsies is justified by the knowledge that this is not a lower airway disease but, as with asthma, the bronchioles are likely to be strongly affected. When compared with the controls, it is clear that considerable changes have occurred particularly with regard to eosinophils which were not present in any of the five controls. The inflammatory infiltrate found in asthmatic and CB patients was similar in this study to results observed previously. In particular, T-cells and macrophages as well as eosinophils were found in increased numbers in comparison with control subjects. Two of the controls had very high neutrophil counts even though they were non-smokers, and may have been in recent contact with some stimulatory agent.

IL-5 was detected in some normal subjects and asthmatics, but was not detected in CB, although eosinophils were present in both asthmatics and CB. Although IL-5 immunoreactivity is not usually found in biopsies from normal subjects, the results of the present study are not related to the selection of the subjects who were not asthmatics nor allergic nor to the antibody used which was highly specific. Moreover, as already reported, eosinophils did not appear to be degranulated in stable CB patients (20) and it is possible that the eosinophilic inflammation in this disease is not directly driven by IL-5.

The most important new finding is the lack of IL-1β and IFN-γ in the CB patients, an observation that needs conformation in larger numbers of samples. Moreover, there were many more CD4+ and CD8+ cells compared with normal controls, particularly the latter, but at the same time there was no staining for IFN-γ. In asthma, Th1-cytokines such as IFN-γ were increased in bronchial biopsies and this finding might be surprising since a Th2-like response has been observed. However, this was more usually observed after allergen challenge which may drive the cytokine pattern towards a Th2-response (12,33). However, in stable asthmatics, it has been found that many T-cells were of the Th1 or Th0 phenotype in the airways (34). Patients in this study have been studied at steady state explaining the importance of IFN-γ immunoreactivity. The low incidence of TNF-α accords with previous data on stable CB patients (21,22) and also adds to the strong suggestion that even though macrophages are clearly present in large numbers, their inflammatory cytokines do not appear to be of great relevance in the disease process. On the contrary, the high numbers of CD8+ T-cells warrants further investigation and a role for these cells needs to be further examined.

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