Neutrophilia and High Level of Interleukin 8 in the Bronchoalveolar Lavage Fluid of Diffuse Panbronchiolitis

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Summary: Diffuse panbronchiolitis (DPB) is a chronic airway disorder exclusively seen in Japan, characterized by chronic inflammation of the respiratory bronchioles leading to air flow obstruction. Although the pathogenesis of the disorder is unknown, current studies based on bronchoalveolar lavage (BAL) have implied an important role of neutrophil-mediated inflammation as a characteristic pathological feature of the disease. Interleukin 8 (IL-8), a cytokine with potent chemotactic activity for neutrophils, has been proven to have close association with many inflammatory disorders. In this study, neutrophil chemotactic activity (NCA) and IL-8 were measured in the BAL fluid in eight patients with DPB to examine the roles of IL-8 in the neutrophil recruitment to the lower respiratory tract in DPB. Significantly higher levels of NCA and IL-8 were demonstrated in the BAL fluid of DPB patients (71.6 ± 3.6%, 491.9 ± 48.0 pg/ml, respectively) compared with that of chronic bronchitis (CB) patients (24.8 ± 2.8%, 54.1 ± 13.9 pg/ml, p<0.001) and of healthy control subjects (7.0 ± 0.9%, 14.2 ± 3.9 pg/ml, p<0.001). The IL-8 values were positively correlated with the NCA in the BAL fluid of DPB patients. Treatment with anti-IL-8 could significantly inhibited the NCA. These results suggest that IL-8 plays an important role in the recruitment of neutrophils to the lower respiratory tract in DPB.

Key words: diffuse panbronchiolitis—interleukin 8—chemotaxis—bronchoalveolar lavage—neutrophil

Introduction

Diffuse panbronchiolitis (DPB) is an obstructive lung disease almost exclusively seen in Japan, characterized clinically by chronic production of purulent sputum, exertional dyspnea and diffuse small nodular shadows on a chest radiogram. As the disease progresses, airway dilatation and air-trapping secondary to bronchiolar obstruction occur. Pathologically, DPB is characterized by a prominent inflammation in the respiratory bronchioles with a cellular infiltration, accompanied by interstitial accumulations of foam cells. Although the pathogenesis of DPB is not known, recent clinical investigations suggested an important role of neutrophils in the pathogenesis of DPB. Bronchoalveolar lavage (BAL) fluid from patients with DPB had an increased number of neutrophils (Ichikawa et al. 1990) and a high level of neutrophil elastase activity (Ninomiya et al. 1992). Sputum of patients with DPB contained a high neutrophil elastase activity (Mikami,
These findings suggest that accumulation and activation of neutrophils in the lower respiratory tract is an important pathophysiological feature of DPB. The mechanism of the neutrophil recruitment to the lower respiratory tract has not been clarified. However, as all of the neutrophils in the respiratory tract are derived from vasculature, neutrophil chemotaxis, a process in which neutrophils migrate toward certain chemical stimuli, must be as an essential component of the pathology in DPB. In this study, BAL fluid from patients with DPB was analyzed in view of assessing neutrophil chemotaxis.

**Methods**

**Subjects**

Eight patients with DPB were investigated. Eight patients with chronic bronchitis (CB) and eight healthy subjects were selected as diseased controls and normal controls, respectively (Table 1). Six of the eight patients with DPB fulfilled the criteria of the Grant-in-Aid from the ministry of Health and Welfare of Japan (Homma, 1976), which are productive cough, shortness of breath, rales on auscultation of the chest, diffusely disseminated small nodular shadows with hyperinflation on a chest radiogram, and decreased forced expiratory volume with or without decreased vital capacity on pulmonary function test. The diagnosis of the remaining two subjects was established pathologically on open lung biopsy. The diagnosis of CB was based on the criteria of the American Thoracic Society (1987).

**Sampling of BAL fluid**

BAL was performed in a segment or subsegment of the right middle lobe with a flexible fiberoptic bronchoscope (Olympus BFIT-20, Japan) as previously described (Ichikawa et al. 1990). Briefly, after local anesthesia of the upper airways with 4% lidocaine, the bronchoscope was passed through the upper airways and the tip was wedged in the bronchus. 150 ml (three 50 ml aliquots) of 0.9% sterile saline was instilled and immediately aspirated via the bronchoscope. The aspirated BAL fluid was strained through a sheet of sterile surgical gauze and centrifuged to separate cellular component from non-cellular component. The cell pellet was washed and resuspended for total cell count. Differential cell counts were performed on May-Giemsa smear preparation of the cells. The supernatant fluid was stored at -70°C until used.

**Chemotaxis assay**

The neutrophilic chemotactic activity (NCA) in the BAL fluid was determined by a modified Boyden chamber method (Harvath et al. 1980). In brief, neutrophils were isolated from blood of a normal

### Table 1.

**Characteristics of the three groups of subjects**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with CAD</th>
<th>Normal Subjects (N=8)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>DPB (N=8)</td>
<td>CB (N=8)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>43.8±6.3</td>
<td>55.0±3.2</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/2</td>
<td>5/3</td>
</tr>
<tr>
<td>No. of Smokers</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

* Plus-minus values are mean±SEM. CAD: chronic airway disease
individual by the sedimentation method (Boyum, 1968) and resuspended in Hank's balanced salt solution at 2×10^6 cells/ml. A 25 µl of samples for chemotaxis assay was seeded in the bottom well of a 48-well microchemotactic chamber (Neuro Probe, Bethesda, MD), which was subsequently covered with a polycarbonate membrane filter sheet with 3 µm holes (25×80 mm) and then with the top plate of the chamber. The wells in the top plate were filled with 50 µl of the neutrophil suspension. Then the chamber was incubated for 30 min at 37 ºC under 5% CO₂ in humidified air. The membrane filter was removed and stained with Diff-Quick® (Harleco, Gibbstown, NJ) for cell count. The cells which migrated through the membrane filter to the other side were counted in ten microscopic high power fields (Original magnification×1000). NCA was expressed as the percentage of a N-formyl-Met-Leu-Phe (fMLP) (10⁻⁶M; Sigma) as positive control. NCA of the BAL fluid of DPB patients was further evaluated in the presence of a rabbit anti human-IL-8 antisera (Upstate Biotechnology Inc., Lake Placid, NY) and of a normal rabbit serum (Antibodies Inc., Davis, CA). NCA was expressed as the number of the migrated cells.

Quantification of IL-8 and albumin

IL-8 was measured in the BAL fluid by enzyme-linked immunoassay (ELISA) using a commercially available kit (Toray-Fuji Bionics Inc., Japan). Briefly, samples were placed in microwells on which primary antibody to human IL-8 was immobilized, and sandwiched between the primary antibody and the enzyme-labeled secondary antibody. Then the substrate for the enzyme was added and the immune complexes formed in the microwells were quantified by spectrophotometry. Albumin concentration in the BAL fluid was determined using radioimmunoassay kit (Shionogi, Japan). To correct the sampling variations during the BAL procedure, the IL-8 values of the BAL fluid were normalized to albumin and expressed as ng/mg albumin for comparison.

Statistical analysis

All values were expressed as mean±SEM. All comparisons were made using unpaired t test. Paired t test was used for the comparison of the NCA between samples from the same subject. Simple linear correlation (Pearson’s r) was employed to evaluate the correlation between NCA and IL-8 values in the BAL fluid. A p value was obtained by two-tailed calculation, and <0.05 was considered significant.

Results

Cell analysis in BAL

BAL results are summarized in Table 2. The total number of cells recovered

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients with CAD</th>
<th>Normal Subjects</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPB</td>
<td>CB</td>
<td>DPB vs CB</td>
</tr>
<tr>
<td>Total cells (×10⁵/ml)</td>
<td>2.0±0.3</td>
<td>1.6±0.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>% of Neutrophils (%)</td>
<td>47.3±6.2</td>
<td>3.6±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils (×10⁹/ml)</td>
<td>102.7±25.1</td>
<td>5.8±1.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Plus-minus values are mean±SEM. N.S.: not significant
from DPB patients \((2.0 \pm 0.3 \times 10^5 / \text{ml})\) was significantly greater than that from healthy controls \((1.0 \pm 0.1 \times 10^5 / \text{ml})\), although there was no difference compared with that from CB patients \((1.6 \pm 0.2 \times 10^5 / \text{ml})\). BAL from DPB patients contained a significantly greater proportion of neutrophils \((47.3 \pm 6.2\%)\) compared with that from CB patients \((3.6 \pm 0.7\%)\) and from healthy controls \((1.0 \pm 0.4\%)\), and the absolute number of neutrophils in BAL fluid was markedly increased in DPB patients \((102.7 \pm 25.1 \times 10^3 / \text{ml})\) compared with other two control groups (CB: \(5.8 \pm 1.3 \times 10^3 / \text{ml}\), healthy controls: \(1.2 \pm 0.4 \times 10^3 / \text{ml}\)).

**NCA in BAL fluid**

BAL fluid of DPB patients \((71.6 \pm 3.6\%)\) showed significantly higher NCA compared with that of CB patients \((24.8 \pm 2.8\%)\) and of healthy controls \((7.0 \pm 0.9\%)\) (Table 3). The NCA in the BAL fluid from DPB treated with anti IL-8 was markedly lower than in the fluid treated with control serum (Fig. 1).

**IL-8 and albumin**

IL-8 value in the BAL fluid of DPB patients \((491.9 \pm 48.0 \text{ pg/ml})\) was signifi-

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TABLE 3.

*Plus-minus values are mean±SEM. N.S.: not significant*
Fig. 2. Correlation between IL-8 and NCA in the BAL fluid from patients with DPB.

Significantly elevated compared with that of CB patients (54.1 ± 13.9 pg/ml) and of healthy controls (14.2 ± 3.9 pg/ml) (Table 3). There was no difference in albumin concentration in the BAL fluid among the three groups. When normalized to albumin concentration, BAL fluid of DPB patients contained significantly higher level of IL-8 (19.1 ± 2.3) compared with that of CB patients (1.4 ± 0.4) and of healthy controls (0.4 ± 0.1). There was no difference in the serum IL-8 between DPB patients (69.2 ± 15.3 pg/ml) and CB patients (48.0 ± 12.7 pg/ml), both of which were significantly higher than healthy controls (15.8 ± 5.1 pg/ml).

Correlation of BAL IL-8 and NCA in patients with DPB

Linear regression analysis demonstrated a significant positive correlation between IL-8 value and NCA in the BAL fluid in patients with DPB (r = 0.86, p < 0.01) (Fig. 2).

Discussion

In addition to its role as the first line of defense against pathogenetic microbes or inhaled noxious particles, neutrophils have recently been implicated as mediators of destruction of normal tissue of the lung. Neutrophils release a wide range of granule derived enzymes, especially proteinases and collagenases, which can degrade the connective tissue of the lung. They also produce oxygen radicals which potentiate the action of the granule-derived enzymes. Neutrophil-mediated inflammation has been implicated in the pathogenesis of several lung diseases: pulmonary emphysema (Janoff, 1983), idiopathic pulmonary fibrosis (IPF) (Hunninghake et al. 1981), adult respiratory distress syndrome (ARDS) (Weiland et al. 1986), and smokers lung (Hunninghake and Crystal, 1983). These disorders have a common pathological feature of accumulation of neutrophils in the lung. Neutrophil-mediated inflammation is also considered as a pathogenesis of DPB, because increased number of neutrophils, as was demonstrated in this investigation, and high level of elastase activity was demonstrated in the BAL fluid of patients with DPB (Ichikawa et al. 1992). Although the mechanism of...
this neutrophil accumulation is not fully understood, it is apparent that chemoattractants play an important role in the migration of neutrophils from the vascular compartment to the site of inflammation. Several kinds of chemotactic factors have been demonstrated in the lung: bacterial products fMLP and C5a (Hopkins et al. 1989) have been established as neutrophil chemoattractants in the pneumatic lungs, and C5a and Leukotriene B4 have been demonstrated as chemoattractant in the BAL fluid from patients with chronic airway diseases including DPB (Ozaki et al. 1992). Recently, a novel neutrophil chemotactic factor has been purified from a culture media of stimulated blood mononuclear cells and referred to as neutrophil activating peptide 1 (Yoshimura et al. 1987) or Interleukin 8 (IL-8). IL-8 is a 72 amino acid polypeptide with potent chemotactic activity at least equal to C5a or fMLP. In addition, IL-8 activates neutrophils to release enzymes derived from azurophilic granules (Schroder, 1989). IL-8 has a longer half life compared with other chemoattractants (Strieter et al. 1989). A role of IL-8 in the pathogenesis of inflammatory disorders, such as rheumatoid arthritis (Seitz et al. 1991) and psoriasis (Schroder and Christophers, 1986), has been established. IL-8 has also been implicated in the pathogenesis of adult respiratory distress syndrome (Donnelly et al. 1993) and idiopathic pulmonary fibrosis (Lynch et al. 1992), in which neutrophils are predominant inflammatory cells. In this investigation, I focused on neutrophil chemotaxis and on the implication of IL-8 as a neutrophil chemotactic factor in DPB. The results showed a significantly increased number of neutrophils and higher level of NCA in the BAL fluid from DPB compared with CB, another type of airway disorder, and with healthy controls. It should be noted that a significantly higher value of IL-8 was detected in the BAL fluid from patients with DPB compared with patients with CB and healthy controls, and that IL-8 value was positively correlated with the NCA of the BAL fluid from DPB group. Furthermore, anti IL-8 could apparently inhibit the NCA in the BAL fluid from DPB group. These results suggested an important role of IL-8 in the neutrophilic accumulation in DPB. There was no difference in the serum IL-8 value between the DPB group and the CB group, both of which were higher than that of healthy subjects. These results implicated that IL-8 was produced in the lung and induced the neutrophilic accumulation.

Although IL-8 had initially been thought of as a chemoattractant derived from blood monocytes, various kinds of cell have been established as the source of IL-8. Not only immune cells but also non-immune cells, like some mesenchymal cells, have been demonstrated to produce IL-8. It is difficult to determine the major source of IL-8 in the BAL fluid, because BAL reflects many components of the lung, including inflammatory cells lining the epithelial surface of the lung, pulmonary fibroblasts and bronchial epithelial cells. However, since DPB mainly affects the airways, bronchial epithelial cells, which have been proven to produce IL-8 in response to inflammatory stimuli (Nakamura et al. 1991), must be an important source of IL-8 in the BAL fluid from DPB patients. It is quite interesting that neutrophil elastase in the BAL fluid from patients with cystic fibrosis, a chronic disorder of the airways in which, like in DPB, neutrophils were the predominant inflammatory cells in the lower respiratory tract, could induce cultured bronchial epithelial cells to release IL-8 (Nakamura et al. 1991). This result suggests an interaction between bronchial epithelial cells and neutrophils, which results in a persistent inflammation in the airways. It is possible that similar
events occur in the lower respiratory tract in DPB.

Although it is apparent that IL-8 is an important chemoattractant in the lower respiratory tract of patients with DPB, other chemoattractants are also likely to take part in this process, especially, C₅α, leukotriene B₄ (LTB₄), and fMLP. C₅α has been suggested as an important neutrophil chemotactic factor in chronic airway disease (Ozaki et al. 1992). LTB₄ (Hopkins et al. 1989) and fMLP, a bacterial product, are well-established potent chemoattractants for neutrophils. In order to elucidate the pathogenesis of persistent inflammation in DPB further, the contributions of these chemoattractants and of the interaction among them for the recruitment of neutrophils should be determined.

References


Schroder, J.M. (1989). The monocyte-derived neutrophil activating peptide (NAP/interleukin 8) stimulates human neutrophil arachi-