Neutrophil Chemotactic Activity in Bronchoalveolar Lavage Fluid Recovered from Patients with Diffuse Panbronchiolitis

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Summary: The present study was aimed at elucidating the role of inflammatory cells in the pathogenesis of the chronic inflammatory changes in the bronchioles of patients with diffuse panbronchiolitis (DPB) and at determining the mechanism for the clinical efficacy of erythromycin (EM) therapy for these patients. For this purpose, neutrophil percentages, neutrophil chemotactic activity (NCA), IL-8 and TNF-α in the bronchoalveolar lavage fluid (BALF) were measured in 9 patients with DPB. Significantly higher neutrophil percentages, NCA and IL-8 concentrations were demonstrated in the BALF from DPB patients than from chronic bronchitis (CB) patients or healthy control subjects. The levels of these indicators of chronic inflammation in the BALF from DPB patients were significantly decreased after EM therapy. TNF-α was elevated in the BALF from both DPB- and CB-patients and was not decreased by treatment of the DPB patients with EM. From the above results, it can be concluded that IL-8, not TNF-α, is the major chemoattractant for neutrophils and that the inhibition of IL-8 production by EM induces the subsequent depression of neutrophil accumulation in the peripheral airways, and consequently, prevents the peripheral airway tissue damage due to accumulated and activated neutrophils.

Key words bronchoalveolar lavage fluid (BALF), neutrophil chemotactic activity (NCA), IL-8, TNF-α, %PMNL, erythromycin (EM)

Introduction

Diffuse panbronchiolitis (DPB) is a disease entity first described by Honma, Yamanaka, Tanimoto and coworkers (1983) that is characterized by a chronic inflammation of the respiratory bronchioles and an infiltration with chronic inflammatory cells. The disease, which develops almost exclusively in the Japanese and Chinese, progresses insidiously and finally results in respiratory failure. The clinical features of the disease are a purulent sputum due to repeated respiratory infections, exertional dyspnea and diffuse small nodular shadows on chest radiograms. Although the precise pathogenesis is not yet known, recent clinical investigations have suggested an important role of neutrophils. Bronchoalveolar lavage fluid (BALF) from patients with DPB demonstrated increased in neutrophil counts (Ichikawa et al. 1990). Empirical treatment with long-term, low-dose erythromycin (EM) has been demonstrated to be efficacious and is the established treatment, although the mode of action...
of EM has not been fully elucidated. The results of experimental and clinical investigations on this mechanism, strongly indicate that the effect of EM depends not on its antimicrobial activity, but on an immunomodifier activity. In this context, an inhibitory effect on neutrophil accumulation in vitro has recently been demonstrated (Goswami et al. 1990) and it has been suggested that the efficacy of EM in the treatment of DPB is due to the inhibition of neutrophil migration.

The present study was aimed at elucidating the role of the neutrophils, which accumulate in the lesions of the bronchioles, in the pathogenesis of DPB. The mode of action of EM in inhibiting the progression of the disease was also examined. For this purpose, the percentages of polymorphonuclear leukocytes (%PMNL) in bronchoalveolar lavage fluid (BALF) obtained from patients with DPB was determined, as well as the neutrophil chemotactic activities in BALF, e.g. the concentration of IL-8 and TNF-α. In addition, the clinical effects of long term, low dose EM on the percentages of neutrophils, chemotactic activities, and concentrations of IL-8 and TNF-α in the BALF were examined.

Materials and Methods

Subjects

Nine patients with DPB were investigated. Eight patients with chronic bronchitis (CB) and 5 healthy subjects served as disease controls and normal controls, respectively.

All 9 DPB patients (6 men and 3 women), aged 18-57 years with a mean of 45, fulfilled the criteria of the Ministry of Health and Welfare of Japan (Honma, 1981). The diagnosis was established in all subjects on the basis of a histopathological examination of transbronchially biopsied lung specimens. Eight patients with CB (7 men and 1 woman), aged 36-70 years with a mean of 50, served as disease controls. The diagnosis of CB was made on the basis of the criteria of the American Thoracic Society (1987).

All were expectorating a considerable amount of sputum for at least several years and had irreversible obstructive disorders of pulmonary function. The 5 healthy control subjects (4 men and 1 woman), aged 31-61 years with a mean of 45, were non-smokers.

Sampling of bronchoalveolar lavage (BAL) fluid

In the 9 DPB patients, BAL was performed twice during infectious exacerbation-free states, before and after low-dose EM therapy (low-dose, 600 mg/day; long-term, 2-16 months; average, 6.8 months).

The BAL procedures were the same as those previously described (Ichikawa et al. 1990). Briefly, the tip of a flexible fiber optic bronchoscope (Olympus BF-1T 20, Olympus Co., Tokyo, Japan) was wedged into a segmental bronchus in the right middle lobe or the left lingula, and 150 ml (three 50 ml aliquots) of 0.9 percent saline solution was sequentially instilled and aspirated after two deep breaths. The aspirated BAL fluid was passed through a sheet of sterile gauze and centrifuged at 800 rpm for 10 min (Cytospin, Shandon Southern Instruments, Sewikley, PA) to separate the cell pellet from the supernatant. The cell pellet was washed with phosphate
buffered saline solution. After counting the total cell number, the cells were stained with May-Giemsa stain, and the cell types were differentiated. The supernatants of the BALF were stored at \(-70 \, ^\circ C\) until use.

**Quantification of IL-8, TNF-\(\alpha\), and albumin**

The concentration of IL-8 in the BALF was measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Toray, Fuji Bionics Inc., Tokyo, Japan). Briefly, samples were placed in micro-wells on which the primary antibody to human IL-8 was immobilized. The samples were subsequently sandwiched between the primary antibody and the enzyme-labeled secondary antibody. The substrate for the secondary antibody enzyme was added, and the immune complexes formed in the microwells were quantified spectrophotometrically.

Similarly, TNF-\(\alpha\) in the BALF was measured by ELISA using a commercially available kit (Medgenics Inc., Belgium).

The albumin concentration in the BALF was determined using a radioimmunoassay kit (Shionogi, Diagnostic Division, Osaka, Japan). To correct for sampling variations during the BAL procedure, the values for IL-8 and TNF-\(\alpha\) in the BALF were normalized to the albumin concentration and expressed as pg/mg albumin to allow for direct comparisons between specimens.

**Chemotaxis assay**

Neutrophils were isolated from the peripheral blood of a healthy individual by a previously described method (Boyum et al. 1968) and resuspended in Hanks balanced salt solution at a density of \(2 \times 10^6\) cells/ml.

The neutrophil chemotactic activity (NCA) in the BALF was determined by a Boyden chamber method (Boydens, 1962), as modified by Harvath et al. (1980). An aliquot of 25 \(\mu l\) of the BALF sample was added to the bottom well of a 48 well microchemotactic chamber (Neuro Probe, Bethesda, MD), which was subsequently covered with a polycarbonate membrane filter sheet (3 \(\mu l\) pore size) and the top plate of the chamber. The wells of the top plate were filled with 50 \(\mu l\) of the neutrophil suspension. The chamber was incubated for 30 min at \(37 \, ^\circ C\) under 5\% CO\(_2\) in humidified air. The membrane filter was then removed and stained with Diff-Quik (Harleco, Gibbstown, NJ) for cell counting. The cells that migrated through the membrane filter to the other side were counted in ten microscopic high-power fields (original magnification, \(\times 100\)).

NCA was expressed as the percentage of that induced by N-formyl-Met-Leu-Phe (fMLP) at \(10^{-6}\)M (Sigma), which was a positive control. The NCA of the BALF from the DPB patients was further evaluated in the presence of rabbit anti-human-IL-8 antiserum (Upstate Biotechnology Inc., Davis, CA). The NCA was expressed as the mean number of migrating cells.

**Statistical analysis**

All values were expressed as mean \(\pm\) SEM. All comparisons were made using the unpaired t-test. The paired t-test was used for comparisons of NCA samples from the same subject.
Results

Total cell counts and percentages of neutrophils in the BALF

Total cell counts in the BALF obtained from DPB patients were elevated, not only before the treatment with EM, but also after the treatment, when compared to the BALF from CB patients and control subjects; although the differences were not statistically significant (Table 1).

The percentages of neutrophils (%PMNL) in the BALF obtained from DPB patients (43.78±8.1) before EM treatment were significantly (P=0.02) higher than those for CB patients (2.71±0.79) and control subjects (1.00±0.32). After EM therapy, the %PMNL in the DPB patients (25.88±11.71) became significantly (P<0.002) smaller (Table 1).

Neutrophil chemotactic activity (NCA) in the BALF

Results of the quantification of NCA among DPB patients, CB patients and control subjects are shown in Fig. 1. The mean NCA (1.60±0.08) in the BALF from DPB patients was significantly (P<0.001) higher than for CB patients (0.35±0.06) and control subjects (0.56±0.10). The mean NCA BALF for DPB patients after EM therapy (0.80±0.10) was significantly (P<0.001) lower than the initial NCA obtained before the therapy (1.60±0.08).

Concentration of IL-8 in the BALF

In DPB patients, the mean IL-8 concentration in the BALF obtained after EM (0.80±0.63) was significantly lower (P<0.05) than that obtained before treatment (6.78±3.00) (Fig. 2). Furthermore, the elevated NCA in the BALF.

<table>
<thead>
<tr>
<th>TABLE 1.</th>
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<tbody>
<tr>
<td>Total cell counts and percentages of neutrophils in the BALF obtained from diffuse panbronchiolitis (DPB) patients, chronic bronchitis (CB) patients, and healthy controls.</td>
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</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Total cell counts (×10³/ml)</th>
<th>Percentages of Neutrophils (%PMNL)</th>
<th>Neutrophil count (×10³/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>diffuse panbronchiolitis before EM treatment</td>
<td>9</td>
<td>4.23±1.6</td>
<td>43.78±8.1*</td>
<td>173.6±88.2</td>
</tr>
<tr>
<td>after EM treatment</td>
<td>9</td>
<td>2.83±1.11</td>
<td>25.88±11.71*</td>
<td>71.07±46.32</td>
</tr>
<tr>
<td>chronic bronchitis</td>
<td>8</td>
<td>1.57±0.31</td>
<td>2.71±0.79*</td>
<td>4.0±1.10</td>
</tr>
<tr>
<td>healthy controls</td>
<td>5</td>
<td>0.8±0.14</td>
<td>1.0±0.32*</td>
<td>0.76±0.36</td>
</tr>
</tbody>
</table>

* P<0.002 compared with the BALF obtained from DPB patients before EM treatment and after EM treatment.
** (**) P=0.002
*** P compared with the BALF obtained from DPB patients before EM treatment and from CB patients.
**** P compared with the BALF obtained from DPB patients before EM treatment and from healthy controls.
Fig. 1. Neutrophil chemotactic activity (NCA) in the BALF from DPB patients, CB patients, and healthy controls. The NCA was expressed as the percentage of that induced by N-formyl-Met-Leu-Phe (fMLP) at 10^{-6}M (Sigma).

*) P<0.001 compared with the BALF obtained from DPB patients before EM treatment.

Fig. 2. Concentrations of IL-8 in the BALF from DPB patients, CB patients, and healthy controls. The IL-8 values from the BALF were normalized to the albumin concentration and expressed as pg/mg albumin.

*) P<0.05 compared with the BALF obtained from DPB patients before and after EM treatment.
from the DPB patients before EM treatment (1.60±0.08) was almost completely abolished by *in vitro* treatment of the BALF with an anti-IL-8 antibody (0.42±0.06) (Fig. 3).

The IL-8 concentrations were below detectable levels in the BALF from CB patients and control subjects.

![Graph](Fig. 3. Reduction of the NCA in the BALF obtained from DPB patients by *in vitro* treatment with anti-IL-8 antibodies. The NCA in the BALF from untreated DPB patients were determined in the presence or absence of anti-IL-8 antibodies.

*) P<0.0001

Concentration of TNF-α in the BALF

The TNF-α concentrations in the BALF from control subjects were extremely low (0.06±0.01) compared to the concentrations from untreated DPB patients (0.35±0.18) and CB patients (0.35±0.02), but the differences between the 3 groups were not statistically significant (Fig. 4). Furthermore, in the patients with DPB, the TNF-α concentration was not changed significantly (before: 0.35±0.18; after: 0.41±0.20) by EM (Fig. 4).

Discussion

Some macrolide antibiotics, such as EM, are able to inhibit the disease progression of DPB and improve the status of the patient. This activity of EM is considered to be due to an ability to modify the *in vivo* immunological responses rather than to antimicrobial activity, because macrolides are not potent enough to eradicate the majority of respiratory pathogens. They are almost inactive against Pseudomonas aeruginosa which
is frequently involved in infectious exacer-
berations and, as a result, is responsible
for an insidious but continuous destruc-
tion of the bronchioles.

EM has been shown to reduce the
bronchial mucus production and secre-
tion (Goswami et al. 1990) and to sup-
press neutrophil chemotaxis (Esterly,
1987). Ninomiya et al. (1991) demon-
strated that the neutrophil count in the
BALF from P. aeruginosa-infected mice
decreased markedly and dose-depen-
dently after treatment of the mice with
EM, but this was not accompanied by a
decrease in number of the organisms.

Neutrophils, which move to and
accumulate at the sites of an infection,
play an important role in host defenses
by phagocytozing and lysing microor-
ganisms. However, when these cells
accumulate in excess and reside longer
in the lesions in an activated state, they
can cause tissue damage by releasing
oxidative and proteolytic substances.

The structure and function of neu-
trophil chemotactic factors (NCF), such
as FMLP, C_{5a}, LTB_{4} and PAF have now
been clarified in detail. IL-8, which is an
8,000 Kda substance released mainly
from monocytes, possesses chemotactic
activities not only for neutrophils but
also for lymphocytes and basophils.

The NCF play a crucial role in the
pathogenesis of the pulmonary lesions
in idiopathic pulmonary fibrosis
(Hunninghaka et al. 1981), adult respira-
tory distress syndrome (ARDS) (Weiland
et al. 1986) and smoking (Hunninghake
and Crystal, 1983), by attracting excess
neutrophils to the lesions. Since the
marked increase in neutrophil count in
the BALF from ARDS patients is always
accompanied by an increase in IL-8 and
also TNF-α; these two chemoattractants
are probably involved in the patho-
genesis of ARDS (Seitz et al. 1991; Jorens

TNF-α is a cytokine which induces
the expression of adhesion molecules
such as ICAM-1 and ELAM-1 on the
surfaces of vascular endothelial cells. As
a result, it induces inflammatory cells to
transfer into the alveolar spaces (Suzuki,
1993). Therefore, the amounts of IL-8
and TNF-α, in relation to the neutrophil
count and NCA in the BALF from DPB
patients were compared to the values for
CB patients and healthy controls.

Both the %PMNL and NCA were
significantly elevated in untreated DPB
patients, as compared to CB patients and
healthy controls, in parallel with the
increase in IL-8. The elevated %PMNL
and NCA from DPB patients were signif-
ically reduced by EM.

On the other hand, the concentration
of TNF-α was only slightly elevated in
untreated DPB patients as compared to
CB patients and healthy controls. In
contrast to IL-8, the slightly elevated
concentration of TNF-α was not reduced
by EM and was not paralleled by the
reduction in %PMNL and NCA.

Thus, the elevation of NCA in the
DPB patients before EM treatment was
mainly due to IL-8 derived from mono-
cytes (Yoshimura et al. 1987). Indeed,
the NCA from the DPB patients before
EM treatment was almost completely
abolished by the in vitro treatment of
the BALF with anti-IL-8 monoclonal anti-
bodies.

The lack of evidence for a direct
participation of TNF-α in the inflamma-
tion of the bronchioles indicates that
there are mutual interactions between
TNF-α and its antagonists, sTNF-1 and sTNF-2 (Smith et al. 1989).

Using purified neutrophil elastase (NE) derived from respiratory epithelial lining fluid (ELF) of patients with cystic fibrosis (CF), Nakamura et al. (1992) suggested a self-perpetuating inflammatory process on the CF bronchial surface. They hypothesized that NE released by neutrophils induces the bronchial epithelium to secret IL-8, which in turn recruits additional neutrophils to the bronchial surface.

The results of the present investigation and other previous reports (Ichikawa et al. 1991) strongly indicate that the beneficial effect of EM on DBP patients is due to the EM-induced reduction of neutrophil accumulation in the lesions of the bronchioles. The neutrophil-derived elastolytic-like activity is reduced when EM subsequently inhibits the production and release of IL-8 by the cells in the bronchioles.

References


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