Neutrophil Chemotactic Factors in Bacterial Pneumonia*

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The influx of neutrophils into the lung is a prominent feature in patients with bacterial pneumonia. Since neutrophils migrate in response to chemotactic factors, chemotactic activity was evaluated in bronchoalveolar lavage (BAL) fluid obtained from 12 patients with bacterial pneumonia and ten normal control subjects. Chemotactic activity was greatly elevated in the BAL fluid of the pneumonia patients compared with control subjects (p<0.01). To partially characterize the chemotactic factors present in the lavage fluid of the patient group, molecular sieve chromatography was performed on the lavage fluid, and at least three peaks of chemotactic activity were identified. Since the molecular weight of the smaller peaks approximated the molecular weight of two known chemotactic factors, C5a and leukotriene B4, these factors were measured in lavage fluid by radioimmunoassay. C5a was detectable in none of the normal subjects but was detectable in four of 14 BAL samples obtained from the patients. Leukotriene B4 was detectable in all subjects and was significantly elevated in the pneumonia patients (552±95 vs 81±16 pg/ml, p<0.01). These findings demonstrate that elevated neutrophil chemotactic activity is present in the lungs of patients with bacterial pneumonia and suggest that C5a and leukotriene B4 may account, at least in part, for this increase.

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**The influx of neutrophils into the alveolar structures is a characteristic finding in patients with bacterial pneumonia.** Current concepts suggest that this neutrophil influx is critical in host defense, since neutrophilic animals or patients demonstrate increased susceptibility to bacterial pneumonia and often succumb owing to an overwhelming proliferation of bacteria. Numerous factors have been described that are capable of mediating neutrophil recruitment into the lung, including bacterial products, humorally derived factors such as the complement peptide C5a, and factors released from cells in the lower respiratory tract.

The present study was designed to determine whether chemotactic factors for neutrophils could be detected in the lungs of patients with bacterial pneumonia by sampling the lower respiratory tract with BAL. The results demonstrate that increased chemotactic activity is present in the lower respiratory tract and suggest that C5a and at least one cell-derived arachidonic acid metabolite, leukotriene B4 (LTB4), may, at least in part, account for this increase in chemotactic activity.

**Material and Methods**

**Patient Population**

Bacterial pneumonia was diagnosed in 12 patients by the following criteria: (1) clinical pneumonia with fever, dyspnea, and productive cough; (2) chest roentgenograms with areas of consolidation; and (3) isolation of a potential bacterial pathogen from sputum and/or BAL fluid. For comparison, ten normal subjects were also evaluated. Each normal subject had normal results of physician examination, chest roentgenogram, and pulmonary function testing.

**Bronchoalveolar Lavage**

To sample the lower respiratory tract, bronchoscopy and BAL were performed in the roentgenographically involved segments in the patients with bacterial pneumonia and from the right middle lobe, right lower lobe, lingula, or left lower lobe of the normal subjects. A flexible fiberoptic bronchoscope (Olympus IT-R or PD-10, Olympus Corporation of America) was introduced orally and passed through the airways into a subsegmental bronchus. Bronchoalveolar lavage was performed in three separate subsegments by sequentially instilling 5-20 ml aliquots followed by immediate aspiration after each aliquot. The initial aliquot, which represents predominately bronchial material, was processed separately from the subsequent 4 aliquots. The final aliquots, which represent predominately alveolar material, were pooled. Since bacterial pneumonia represents predominately an alveolar inflammation, only the final 4 aliquots were studied.

The BAL fluid was processed similar to previously described methods. Briefly, the fluid was filtered through sterile nylon mesh to remove large particles of mucus. Cell number was evaluated in a hemacytometer and viability of the cells assessed by the ability to exclude trypan blue. Each lavage fluid was evaluated for differential count using a cytocentrifuged cell preparation (Shandon Cytospin II; Shandon Southern). The cell pellets were stained with
a modified Wright's stain (Diff-Quik, American Hospital Supply, Inc) and a differential count performed on 200 cells. To separate the fluid from the cells, the remaining fluid was centrifuged (500 × g, 5 min) and the supernatant removed and frozen at −80°C until studied.

Assay of Neutrophil Chemotactic Activity

The neutrophil chemotactic activity of the bronchoalveolar lavage fluid was evaluated utilizing a blindwell technique. Briefly, 26 µl of each lavage fluid were placed in the bottom well of a 48 well microchemotaxis chamber (Neuro Probe, Inc) and covered with a 3-µ pore membrane (NMF-3-2580 PVPF, Neuro Probe). Neutrophils were isolated for the chemotaxis assay from a single volunteer by dextran sedimentation. The cells were suspended in Hanks' balanced solution with 2 percent bovine serum albumin at 3 × 10⁶ cells/ml and 50 µl of the neutrophil suspension were placed in the top well of the chamber. The chambers were incubated for 20 min at 37°C in 5 percent CO₂, and subsequently the membranes were removed from the chemotaxis chamber, and the neutrophils on the upper side of the membrane were removed by scraping. The cells were stained (Leukostat, Fisher Scientific, Inc) and mounted on glass slides. Chemotactic activity was quantified by counting the number of cells by light microscopy (1,000×) in ten randomly selected fields by a blinded observer and the results expressed as the percentage of the chemotactic response to a 1:10 dilution of zymosan-activated serum.

Fractionation of BAL Fluid by Molecular Sieve Chromatography

Partial characterization of the chemotactic activity present in the BAL fluid was done by molecular sieve chromatography. This was accomplished by fractionating the lavage fluid using high-performance liquid chromatography on a protein PAK 300 column (30 × 0.75 cm, Waters Chromatography) with a mobile phase of phosphate-buffered saline solution at a flow rate of 1 ml/min. Fractions were collected at 25-s intervals and each fraction was assayed for chemotactic activity by the methods described above. Human C5 (180,000 daltons, Sigma), α₁-antiprotease (54,000 daltons, Sigma), ovalbumin (43,000 daltons, Sigma), human neutrophil elastase (29,000 daltons, Sigma), C5a (15,000 daltons, Upjohn Diagnostics) and prostaglandin F₂ (457 daltons, Sigma) were used as molecular weight standards to calibrate the column under identical chromatographic conditions.

Quantification of C5a

The BAL fluid was evaluated for the presence of C5a by radioimmunoassay (Upjohn Diagnostics) following the methods of Hugi and Chenoweth. The useful range of this assay is 10 to 400 ng/ml.

Quantification of Leukotriene B₄

LTB₄ was quantified by radioimmunoassay. First, the LTB₄ was extracted from 0.3 ml of lavage fluid by lowering the pH of the lavage fluid to 3 to 4, using 1.0N HCl and extracted twice with 0.6 ml of ethyl acetate. The ethyl acetate extractions were combined and evaporated to dryness under a nitrogen stream. The extracted LTB₄ was then dissolved in 0.1 ml of 50 mM TRIS-HCl, pH 8.6, with 0.1 percent gelatin and LTB₄ quantified by radioimmunoassay (Amersham, Inc) according to the methods of Salmon et al.

Determination of Epithelial Lining Fluid Concentration of C5a and Leukotriene B₄

Since BAL represents a variable dilution of the fluid lining the surface of the epithelial surface of the alveoli, the concentrations of C5a and LTB₄ were expressed as µg or ng/ml of epithelial lining fluid. This was done by measuring the concentrations of urea in both lavage fluid and plasma and calculating the volume of epithelial lining fluid recovered by BAL according to previously published methods.

Statistical Analysis

All results were analyzed using the Wilcoxon rank sum test. Significance was defined as p < 0.05.

RESULTS

Patient Characterization and BAL Findings

The characteristics of the patients with pneumonia are presented in Table 1. The patients were significantly older than the normal subjects (65 ± 17 vs 30 ± 6 years, p < 0.01). Further, all the patients with pneumonia had underlying conditions that complicated their pneumonia, including six with COPD and one each with head trauma, emphysema, diabetic ketoacidosis, Parkinson's disease, congestive heart failure, fracture of the hip, and a seizure disorder.

Bronchoalveolar lavage was performed without complication in each patient with pneumonia and each normal volunteer. In contrast to the pneumonia patients, the lavage fluid from the normal volunteers was

<table>
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<tr>
<th>Patient</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Complicating Diagnosis</th>
<th>Organism</th>
<th>Total Cells Recovered (× 10⁶)</th>
<th>Concentration of Cells Recovered (10⁶/ml)</th>
<th>Percentage Total Neutrophils Recovered</th>
<th>Neutrophils Recovered (× 10⁶)</th>
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<td>44</td>
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<td>F</td>
<td>COPD</td>
<td>P aeruginosa</td>
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<td>4c</td>
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<td>S aureus</td>
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* Bronchoalveolar lavage was performed on patient 4 on three separate occasions.

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Table 1 — Clinical and Bronchoalveolar Lavage Characteristics of Patients with Bacterial Pneumonia

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sterile. The bronchoscopy was performed in each of the pneumonia patients zero to seven days after the diagnosis of pneumonia for either diagnostic or therapeutic purposes, with one patient (patient 4) undergoing BAL three times for the therapeutic removal of mucoid secretions. The recovery of fluid was variable in the pneumonia patients, ranging between 12 and 75 percent of the amount instilled, but did not differ significantly compared with the normal subjects (data not shown, p>0.2). The total number of cells recovered was significantly larger in the pneumonia patients than in normal subjects, expressed either as total number of cells recovered (85 ± 71 x 10^6 cells vs 37 ± 16 x 10^6 cells, p<0.05) or as cells per milliliter of recovered lavage fluid (1.0 ± 0.7 x 10^6 cells/ml vs 0.25 ± 0.21 x 10^6 cells/ml, p<0.01), but was not larger when expressed as cells per milliliter of epithelial lining fluid (29 ± 18 cells/ml vs 16 ± 14 cells/ml, 0.05<p<0.1). The number of neutrophils was strikingly elevated in the pneumonia patients’ lavage fluid when expressed either as the percentage of cells recovered (80 ± 21 percent vs 4 ± 2 percent, p<0.01); total neutrophils recovered (69 ± 64 x 10^6 cells vs 1.8 ± 3.0 x 10^6 cells, p<0.01); neutrophils per milliliter of recovered lavage fluid (0.84 ± 0.69 x 10^6 cells/ml vs 0.012 ± 0.023 x 10^6 cells/ml, p<0.01), or as neutrophils per milliliter of epithelial lining fluid (22.1 ± 14.8 x 10^6 cells/ml vs 0.87 ± 1.33 cells/ml, p<0.01). There was no correlation between the age, sex, complicating diagnosis and organism identified, with any of the BAL parameters including the percentage of lavage fluid recovered, total cells recovered, or numbers of neutrophils.

Chemotactic Activity of BAL Fluid

The chemotactic activity of BAL fluid obtained from patients with bacterial pneumonia was significantly larger than that of the normal subjects (Fig 1, 110 ± 42 percent vs 41 ± 10 percent of zymosan activated serum, p<0.01). The activity was shown to be chemotactic rather than chemokinetic by checkerboard analysis (data not shown).

Partial Characterization of Neutrophil Chemotactic Activity

To determine the approximate molecular weight of the neutrophil chemotactic activity present in the BAL fluid of patients with bacterial pneumonia, molecular sieving of the BAL was performed using high-performance liquid chromatography. The results from three separate patients with the highest chemotactic activity in their lavage fluid demonstrated multiple peaks of chemotactic activity (Fig 2). There was a peak of chemotactic activity of large molecular weight (estimated mol wt≥54,000 daltons), which is similar to that previously identified in BAL obtained from normal subjects.21 There were two additional peaks of activity, which were estimated to be 12,000 and 29,000 daltons and <500 daltons.

C5a in BAL Fluid

Since a chemotactic peak was identified which was approximately compatible with the molecular weight of C5a (15,000 daltons), the BAL fluids were assayed for C5a using a radioimmunoassay. The results demonstrated that C5a was detectable in four of the lavage fluids from the pneumonia patients but in none of the normal volunteers (Fig 3). There was no correlation between the C5a levels and the clinical or BAL characterization of the four pneumonia patients with detectable C5a including age, complicating diagnosis, organisms recovered, percentage of neutrophils, total neutrophils recovered, or concentrations of neutrophils (p>0.05, all comparisons). There was no significant difference between those patients with detectable

![Chemotactic Activity of BAL Fluid](image-url)
Leukotriene B4 in BAL Fluid

The detection of low molecular weight chemotactic activity in BAL fluid and the observation that both activated alveolar macrophages and neutrophils can release the potent neutrophil chemotactic factor, LTB4, suggest that LTB4 might be present in increased quantities in lavage fluid obtained from the pneumonia patients. Measurement of LTB4 by radioimmunoassay demonstrated an increase in the LTB4 levels in BAL fluid obtained from the pneumonia patients compared with the normal subjects whether expressed as pg/ml of lavage fluid (Fig 4, panel A, p<0.01) or, using urea to estimate dilution during the lavage procedure, as ng/ml of epithelial lining fluid (Fig 4, panel B, p<0.02). The LTB4 levels in the lavage fluid and the epithelial lining fluid of the pneumonia patients did not correlate with any of the clinical or BAL parameters examined including age, complicating diagnosis, organisms recovered, total cells recovered, concentration of cells recovered, percentage of neutrophils, total neutrophils recovered, concentration of neutrophils or chemotactic activity (p>0.05, all comparisons). Although three of the five patients with the largest LTB4 levels in epithelial lining fluid had detectable C5a, with the numbers available there was no correlation between LTB4 and C5a levels in BAL fluid (r = 0.33, p>0.05) or in epithelial lining fluid as estimated by urea (r = 0.87, p>0.05).

FIGURE 2. Chemotactic activity of BAL fluid obtained from three patients with bacterial pneumonia after fractionation of the lavage fluid by molecular sieve chromatography using high performance liquid chromatography. Each panel represents a patient; patient numbers corresponds to those in Table 1. Chemotactic activity expressed as percentage of zymosan activated serum is on the ordinate in each panel; elution volume from column in milliliters is on the abscissa.

C5a and those without detectable C5a in the neutrophil percentage, total neutrophils, neutrophil concentration, or chemotactic activity of the BAL fluid (p>0.2, all comparisons).

FIGURE 3. C5a levels in BAL fluid (panel A) and the estimated levels in the epithelial lining fluid (panel B) of normal subjects and patients with bacterial pneumonia. Panel A: C5a levels expressed (ng/ml) on ordinate, patients groups on abscissa. Shaded area, minimal detectable dose of C5a (10 ng/ml). Panel B: C5a levels in epithelial lining fluid calculated using urea technique. C5a levels (μg/ml) are on ordinate, patient groups on abscissa. Shaded area, estimated minimal detectable dose of C5a.

Discussion

An influx of neutrophils into the alveoli is a characteristic pathologic finding in bacterial pneumonia. Since current concepts suggest that neutrophils migrate to chemotactic factors and that neutrophils are critical to the host in destroying and clearing these
pathogenic bacteria,\textsuperscript{11,12} the mechanisms which cause the migration of neutrophils from the vasculature into the involved alveoli are important in understanding the host defenses against bacterial pneumonia. In this context, the present study examined the lower respiratory tract of patients with bacterial pneumonia by BAL. Marked increases in both the absolute number and percentage of neutrophils recovered confirms histologic findings of neutrophils within the alveolar spaces of the lung in bacterial pneumonia. Furthermore, an increase in neutrophil chemotactic activity was present in the BAL fluids of patients with bacterial pneumonia and two potent neutrophil chemotactic factors, C5a and leukotriene B4 (LTB4), were identified in the lavage fluid. These factors may account, in part, for the increase in chemotactic activity which leads to neutrophil recruitment.

Activation of the complement system leads to cleavage of the fifth component of complement, C5, and one of the cleavage products of C5, C5a, is a potent neutrophil chemotactic factor.\textsuperscript{27,28} Since bacteria and their products can activate the complement system,\textsuperscript{29} the presence of C5a in the lower respiratory tract of patients with bacteria pneumonia would be anticipated. However, other lung disorders, including the adult respiratory distress syndrome,\textsuperscript{31,30} and cystic fibrosis,\textsuperscript{32} have been associated with C5a in the lower respiratory tract, suggesting that this mechanism of neutrophil recruitment is not unique to bacterial pneumonia. In this regard, the generation of C5a at sites of bacterial pneumonia would likely represent a mechanism that would lead to a localized accumulation of neutrophils, rather than the more generalized neutrophilic influx seen in the adult respiratory distress syndrome or cystic fibrosis.

The release of neutrophil chemotactic factors by the resident lung phagocyte, the alveolar macrophage, has been proposed to be an important mechanism for recruiting neutrophils into the lung.\textsuperscript{32-38} Since bacterial products and activated complement components can activate macrophages to release neutrophil chemotactic activity,\textsuperscript{35-38} this likely represents one important mechanism of neutrophil recruitment. LTB4, a product released by both alveolar macrophages and neutrophils,\textsuperscript{32-36} is a potent neutrophil chemotactic factor, and its demonstration in increased quantities in BAL obtained from pneumonia patients suggests this factor represents another important mechanism of neutrophil recruitment in bacterial pneumonia. However, other chemotactic factors released by alveolar macrophages and/or neutrophils including platelet activating factor,\textsuperscript{36,40} tumor necrosis factor, and 12-I hydroxy 5, 6, 10, 14-eicosatetraenoic acid\textsuperscript{41} may also be important.

Numerous potential sources of chemotactic activity are present in the lung. These include chemotactic factors derived from cells other than neutrophils and macrophages,\textsuperscript{42-44} humoral factors other than C5a,\textsuperscript{45-46} and even products released by the invading organism itself.\textsuperscript{47-49} In this regard, many small peptides derived from bacterial proteins that have a formulated amino terminus can function as potent neutrophil chemoattractants. Thus, the finding of several fractions of chemotactic activity in BAL fluid after molecular sieving would be expected. Which of these chemotactic factors are quantitatively most important in recruiting neutrophils cannot be determined from the present data, although the observation that the molecular weight of two of the fractions after molecular sieving closely correspond to the molecular weights of C5a and LTB4 suggests that these factors are likely important.

Bacterial pneumonia represents a clinical syndrome caused by many potential pathogenic organisms. Although the host response may often differ depending on the organism and the clinical status of the patient, the present study demonstrates that in many patients neutrophil recruitment by C5a and LTB4 may be a frequent mechanism of attracting neutrophils to the lung.

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*Figure 4. Leukotriene B4 (LTB4) levels in BAL fluid (panel A) and estimated levels in epithelial lining fluid (panel B) obtained from normal subjects and patients with bacterial pneumonia. Panel A: LTB4 levels expressed (pg/ml) on ordinate, patient groups on abscissa. LTB4 levels significantly elevated in the pneumonia patients vs normal subjects (p<0.01). Panel B: LTB4 levels (ng/ml) on ordinate, patient groups on abscissa. LTB4 levels significantly elevated in pneumonia patients vs normal subjects (p<0.02).*
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