Chemotactic Factors in Bronchial Secretions of Cystic Fibrosis Patients

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To understand chronic neutrophil attraction into cystic fibrosis airways, both global chemotactic activity and individual chemotactic factors were studied in bronchial secretions. Bronchial secretions of 8 cystic fibrosis patients, collected on the first day of admission for antibiotic treatment, showed a high chemotactic index (19.4 ± 5.7, $n = 8$). Fractionation by gel filtration of bronchial secretions resulted in three chemotactic fractions. The first factor corresponded to interleukin-8, and the second activated neutrophils via the FMLP receptor. The third factor, which was of lower molecular weight, did not activate FMLP or leukotriene B_4 receptors, and its nature is still under investigation. Treating patients with antibiotics reduced global chemotactic activity, mainly by reducing the activity due to stimulation of the FMLP receptor.

Patients with cystic fibrosis (CF) suffer from a progressively destructive bronchitis that leads to respiratory failure. Chronic infection is characterized by airway colonization with bacteria (e.g., *Haemophilus influenzae, Staphylococcus aureus,* and *Pseudomonas aeruginosa*) totalling up to 10^9–10^{11} microorganisms and by the presence of 10^8 neutrophils/mL of bronchial secretions [1, 2].

Since the damage to airway walls observed in CF is thought to be mainly mediated by neutrophil proteases [3, 4], the identification of chemotactic agents may be important for improving therapeutic strategies. The mechanisms involved in the chronic immigration of neutrophils into the airways are not well understood. Several different chemotactic factors have been found in bronchial secretions, among them the anaphylatoxin C5a [5], interleukin (IL)-8 [6], and leukotriene B_4 (LTB_4) [7]. Other factors, such as bacterial chemotactic peptides and degradation products derived from the extracellular matrix protein elastin or from the complex between elastase and its inhibitor α1-antiprotease [8], are thought to be present. The relative importance of these factors is not known, nor is it known which factors are present simultaneously in bronchial secretions or how the levels of chemotactic factors in bronchial secretions are regulated by therapeutic measures.

The aims of this study were to measure the global chemotactic activity of CF bronchial secretions and to characterize the chemotactic factors by use of separation by gel filtration. Since intravenous antibiotic treatment was shown to decrease the concentration of bacterial pathogens in bronchial secretions and to decrease the number of inflammatory cells [9], the chemotactic activity before and after intravenous antibiotic therapy was also studied.

Patients and Methods

Patients. Eight CF patients (12–46 years old) who were hospitalized in the University Hospitals of Geneva between 1992 and 1994 were included in this study. The diagnosis of CF was confirmed by a sweat test and genetic analysis. All patients were colonized with *Pseudomonas* species and treated at regular intervals with a combination of an aminoglycoside and a cephalosporin.

Bronchial secretions. Bronchial secretions were collected over 24 h on the first and last days of antibiotic treatment. Sputum samples were stored at 4°C during the collection period, weighed, and processed as previously described [3].

Chemotactic activity. Chemotactic activity was determined by a modified Boyden chamber assay as described [10]. In a modification of this method, neutrophils were quantified by a viability test based on a colorimetric determination of the cleavage of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WTS-1; Boehringer Mannheim, Mannheim, Germany). Results were expressed as the chemotactic index, which was defined as the ratio between the total migrated neutrophils and the number of neutrophils migrating nonspecifically. All assays included as positive controls 10^{-6} *M* FLMP (Sigma, St. Louis) and 7.6 n*M* recombinant human IL-8 (rhIL-8, 72 amino acids; from A. Proudfoot, GLAXO Molecular Biology Institute, Geneva). Tests whose results were to be compared were run the same day with one batch of neutrophils.

To assess chemotactic activity due to IL-8, we preincubated samples for 16 h at 4°C with a monoclonal antibody against rhIL-8 (2 μg/mL; Innogenetics, Zwijndrecht, Belgium). To assess the chemotactic activity due to activation of the neutrophil receptor for FMLP or for LTB_4, we preincubated neutrophils for 10 min at 37°C with 500 μM butyloxycarbonyl-methionyl-leucyl-phenylalanine (BocMLP, Sigma) or with 1 μM Ly288535 (Lilly Research, Indianapolis), which are antagonists of the two receptors,

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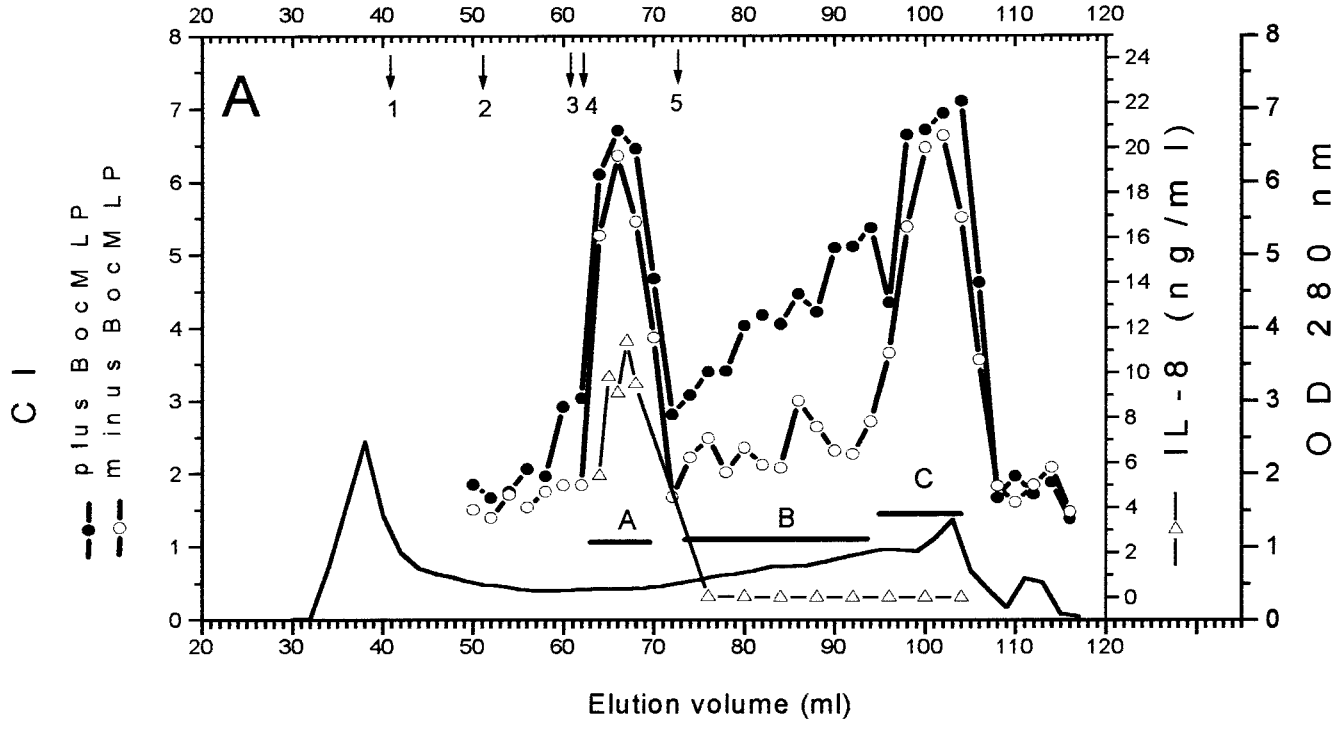

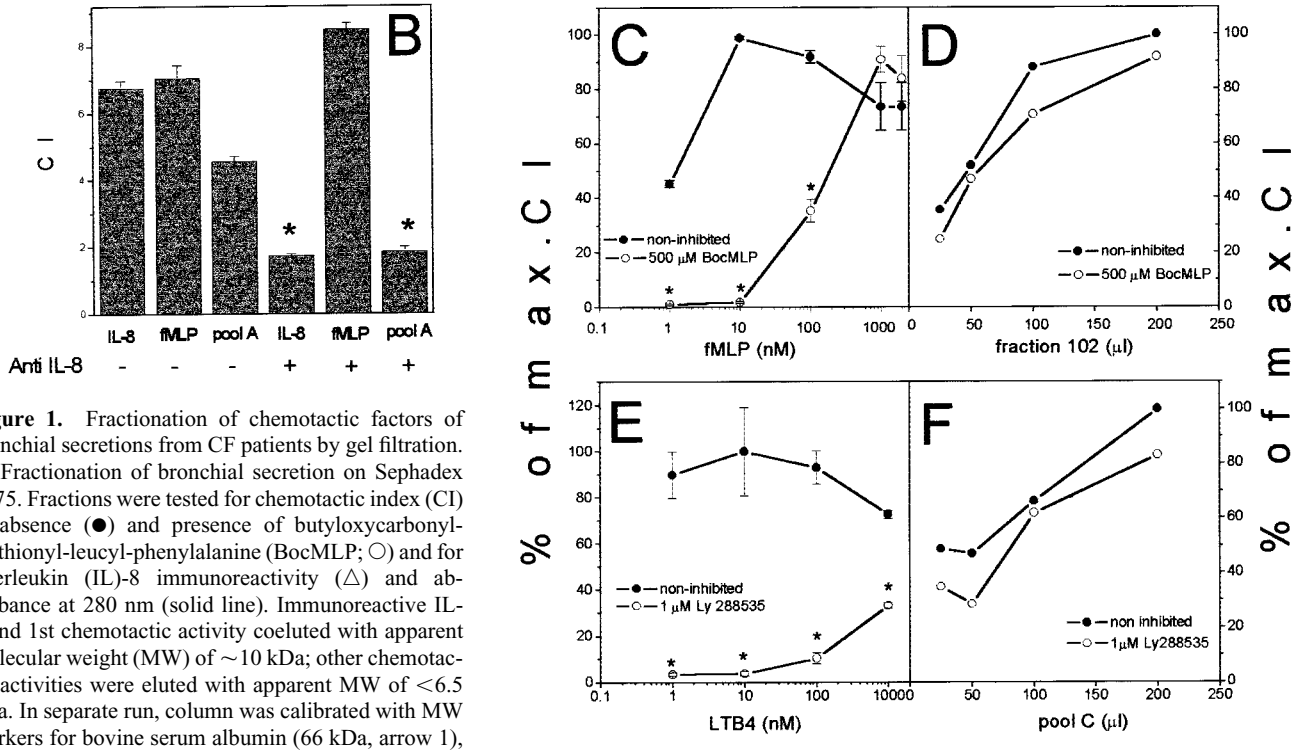

Figure 1. Fractionation of chemotactic factors of bronchial secretions from CF patients by gel filtration. **A**, Fractionation of bronchial secretion on Sephadex G-75. Fractions were tested for chemotactic index (CI) in absence (●) and presence of butyloxycarbonyl-methionyl-leucyl-phenylalanine (BocMLP; ○) and for interleukin (IL)-8 immunoreactivity (△) and absorbance at 280 nm (solid line). Immunoreactive IL-8 and 1st chemotactic activity coeluted with apparent molecular weight (MW) of ~10 kDa; other chemotactic activities were eluted with apparent MW of <6.5 kDa. In separate run, column was calibrated with MW markers for bovine serum albumin (66 kDa, arrow 1), carbonic anhydrase (29 kDa, arrow 2), cytochrome C (12.4 kDa, arrow 3), recombinant human IL-8 (rhIL-8, arrow 4), and aprotinin (6.5 kDa, arrow 5). Fractions were pooled into pools A, B, and C (indicated by horizontal solid bars). **B**, Anti–IL-8 antibody inhibition of chemotactic activity of pool A, rhIL-8 (7.6 n*M*), and FMLP (10 n*M*). **C**, Chemotactic activity of FMLP in absence or presence of BocMLP. **D**, Chemotactic activity of fraction 102 of elution of Sephadex G-75 at different doses in absence or presence of BocMLP. **E**, Chemotactic activity of leukotriene B_4 (LTB_4) in absence or presence of receptor inhibitor Ly288535. **F**, Chemotactic activity of pool C in absence and presence of Ly288535. **B, C, E**, Mean ± SE is shown of triplicate determinations. **A–F** show 1 of at least 3 experiments. * Values for which *P* is <.05 compared with noninhibited controls. max = maximum.

respectively. LTB_4 was from Biomol (Plymouth Meeting, PA). In control experiments, neutrophils were preincubated with DMSO at the same dilution. Neutrophils preincubated with BocMLP or Ly288535 retained full chemotactic activity toward chemotactic factors acting via different receptors (not shown).

IL-8 concentrations. IL-8 concentrations were determined by ELISA from Innogenetics or from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam).

Gel filtration chromatography. Gel filtration chromatography samples were heated at 95°C for 15 min, centrifuged, and passed through filters with 0.22-μm pores before being subjected to fractionation on a 1 × 120–cm Sephadex G-75 (Pharmacia, Uppsala, Sweden) gel chromatography column. The column was eluted with PBS at 4 mL/h, and 1-mL fractions were collected. Pools from the elution were concentrated by vacuum centrifugation and applied to a 1 × 120–cm Sephadex G-25 column, which was eluted with PBS at 10 mL/h. In separate runs, the columns were calibrated with molecular weight standards.

Statistical analysis. Samples from 8 patients were assessed twice (before and after administration of antibiotics) as paired samples by use of Wilcoxon's signed rank test. A *t* test for nonpaired samples was used where applicable for inhibition experiments. $P < .05$ was considered significant. Values were expressed as mean ± SE.

Results

Sputum samples that were collected from 8 patients at hospital admission were chemotactically active and showed a chemotactic index of 19.4 ± 5.7. This activity was higher than the maximal chemotactic activity of strong chemotactic factors such as FMLP or IL-8 (not shown). Heating the samples to 95°C for 15 min allowed most of the proteins to coagulate and to destroy proteolytic activity, while chemotactic activity was not changed (not shown).

A sputum sample collected at admission from 1 of the 8 patients was precleared by heat treatment and centrifugation and subjected to gel chromatography on Sephadex G-75. Figure 1A shows the elution pattern of this column. The large proteins eluted in the void volume. IL-8 eluted in fractions 63–70, while rhIL-8 eluted at fraction 63 (figure 1A, arrow 4). The first peak of chemotactic activity (pool A, figure 1A) coeluted with IL-8 in fractions 63–70. This activity was inhibited by a monoclonal antibody directed against rhIL-8, as was that of rhIL-8, while the chemotactic activity of FMLP was not inhibited (figure 1B).

The second chemotactic activity eluted in fractions 75–104 and was clearly separated from IL-8. It formed a plateau (fractions 75–94, pool B) and a sharper peak at fractions 98–104 (pool C). The factors constituting pool B and pool C seemed to be quite heterogeneous. The chemotactic activity of pool B was inhibited by BocMLP, whereas that of the fractions of pool C was not (figure 1A). Since inhibition could be missed when the concentration of the chemotactic factor is too high, as shown for FMLP at 1 μM (figure 1C), BocMLP inhibition was tested on different concentrations of the chemotactic material of a fraction of pool C. As shown in figure 1D, BocMLP did not inhibit the activity of fraction 102 at any concentration tested. No immunoreactivity of IL-8 (<1 p*M*) was found in pool C (figure 1A), nor was the chemotactic activity inhibited by the antibody against IL-8 (not shown). Furthermore, chemotactic activity of pool C was not inhibited by the LTB_4 receptor antagonist Ly288535 at any concentration tested (figure 1F). Taken together, these results indicate that pool C contains a chemotactic activity that does not act via either the IL-8 receptor, the FMLP, or the LTB_4 receptor when attracting neutrophils in our chemotaxis assay.

In the elution of the Sephadex G-75 column, the FMLP-like factors (pool B) were not well separated from pool C; thus, the fractions corresponding to the two pools were combined from three runs on Sephadex G-75, concentrated, and applied to a Sephadex G-25 column, in which they could be completely separated (not shown).

To confirm the results obtained with the bronchial secretions from 1 patient, it was necessary to investigate more samples. Bronchial secretions collected at admission were subjected to chromatography on Sephadex G-75. When all elutions were compared, three major patterns emerged (figure 1A, 2A, and 2B). Three bronchial secretions contained no detectable chemotactic activity due to IL-8 or to FMLP, since all activity eluted at a position corresponding to pool C (figure 2A). Two bronchial secretions resolved their chemotactic activity into two peaks, one due to IL-8 and a second one corresponding to pool C in figures 1A and 2B. Three bronchial secretions contained all three chemotactic factors, as did the one shown in figure 1A. No other chemotactic factors were detected in any of the samples (not shown).

To evaluate the influence of antibiotic treatment, the chemotactic activities before and after treatment were compared in one treatment course of each of the 8 patients. Global chemotactic activity decreased significantly from a chemotactic index of 19.4 ± 2.0 to 11.4 ± 1.3 ($n = 8$, $P = .05$) after treatment, which is still very high. IL-8 concentrations did not change significantly in the same time period (not shown).

To determine the source of the decrease of the overall chemotactic activity after treatment, bronchial secretions collected after each of the eight treatment courses were fractionated on Sephadex G-75. The results showed that antibiotic treatment reduced chemotactic activity mainly by lowering the amount of FMLP-like material in bronchial secretions. The activity corresponding to pool C remained unchanged.

Discussion

The high chemotactic activity of bronchial secretions is due to chemotactic factors that act together in neutrophil attraction. Since most known chemotactic factors have biphasic dose-response relationships, the analysis of the relative importance of single factors in unfractionated bronchial secretions with specific inhibitors is unreliable or may even lead to false con-

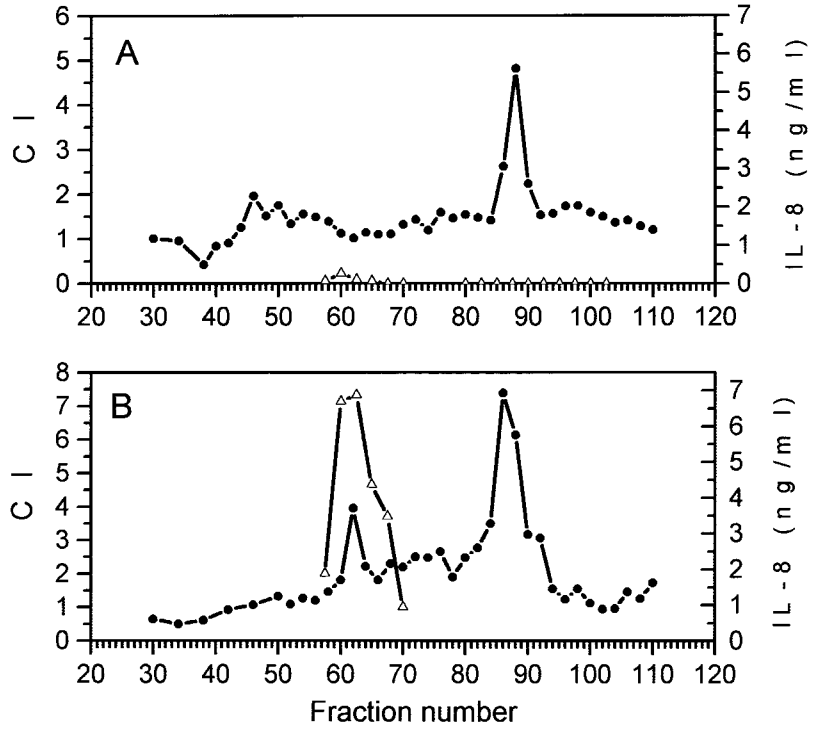

Figure 2. Elution of chemotactic activity of bronchial secretions obtained from 8 patients before antibiotic treatment. Samples were chromatographed on Sephadex G-75 (Pharmacia, Uppsala, Sweden). Shown are 2/3 typical elution patterns; 3rd pattern is shown in figure 1A. **A**, Elution of bronchial secretion that contains only chemotactic activity corresponding to pool C (figure 1A). **B**, Elution of bronchial secretion that contains chemotactic activity due to interleukin (IL)-8 and corresponding to pool C. Neither sample contained detectable activity due to formyl-methionyl-leucyl-phenylalanine–like material. ● = chemotactic activity, △ = IL-8 as determined by ELISA, CI = chemotactic index.

clusions. The fractionation proposed in this study revealed three major chemotactic factors in bronchial secretions. Their relative importance varied greatly when samples of bronchial secretions from different patients were compared.

Since the activity of pool A was totally inhibited by anti–IL-8 antibody (figure 1B), we concluded that this activity was entirely due to IL-8 and that no other chemotactic factor of the size of IL-8, such as C5a and GROα, was present after the elution.

The chemotactic activity of pool B (figure 1A) was inhibited by BocMLP, a specific antagonist of the FMLP receptor. Since this activity was spread over many fractions, both on Sephadex G-75 and on Sephadex G-25, it seemed to be very heterogeneous in molecular size. Compared with the elution of FMLP, which eluted at a fraction corresponding to the end of the elution of pool B from Sephadex G-25, the FMLP-like material was mostly composed of molecules larger than FMLP and therefore very much resembled the chemotactins found in culture supernatants of *Pseudomonas aeruginosa* as described by Fontan et al. [11]. It is thus likely that chemotactic factors from pool B are of bacterial origin. The fact that they were not found in all patients was surprising since all patients were colonized by *Pseudomonas* species (figure 2). Furthermore, the 4.2-kDa fragment of the α1-proteinase inhibitor, described as being chemotactic [8], would also be found in pool B if present. However, since almost all of the activity in pool B was inhibited by BocMLP, the fragment of the α1-proteinase inhibitor does not seem to be a major chemotactic factor in bronchial secretions.

The chemotactic activity of pool C (figure 1A), although not completely separated on Sephadex G-75, could be separated from pool B on Sephadex G-25. Its activity eluted like very small molecules and was due neither to FMLP-like material nor to IL-8 degradation products, and it was not due to LTB_4 (figure 1). Future analysis will show whether it is identical to one of the candidate chemotactic factors described, such as trinucleotides (ATP or UTP) [12], fungal products [13], or oxidation products of lipids, such as 4-hydroxynonenal [14].

Antibiotic treatment was expected to reduce the level of FMLP-like bacterial products and, as a consequence of lowering infection, the level of IL-8. Samples collected before and after treatment showed that reducing the bacteria load with antibiotics had an effect on the bacterial chemotactic factors (pool B) and to a small extent on IL-8 concentration (not shown). In contrast to our study, in which patients were not in exacerbation, Richman-Eisenstadt et al. [6] found a decrease of IL-8 after antibiotic treatment in all of their 5 patients who were in exacerbation, suggesting that in an acute infection, IL-8 concentration was more susceptible to antibiotics.

In summary, we have shown that by fractionation of bronchial secretions of CF patients with chronic airway inflammation, three different chemotactic factors could be distinguished. This analysis may serve as a basis for future investigations of therapeutic approaches aimed at lowering chemotactic activity in bronchial secretions and controlling inflammation and tissue destruction.

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