Characterization of a Novel Chemotactic Factor for Neutrophils in the Bronchial Secretions of Patients with Cystic Fibrosis

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Chronic airway inflammation is a hallmark of cystic fibrosis (CF). Biological products with chemotactic activity are essential for neutrophil recruitment to sites of inflammation. The presence of a factor with chemotactic activity higher than that of interleukin (IL)–8 in the bronchial secretions of patients with CF has recently been reported. This article reports that the chemotactic activity of this factor remained unaffected by a variety of physical treatments and could be distinguished from those of IL-8, formylmethionylleucylphenylalanine, leukotriene B4, and platelet-activating factor. The factor induced chemotaxis and chemokinesis of neutrophils, and its chemotactic activity was sensitive to pertussis toxin and thapsigargin. Semipurified preparation of the chemotactic factor increased transiently intracellular Ca^{2+} concentration but failed to stimulate the release of neutrophil primary granules and the production of superoxide, suggesting that the semipurified chemotactic factor is a Ca^{2+}-dependent chemotactic agent.

In several clinical studies, an increased concentration of IL-8 and high numbers of neutrophils in the bronchoalveolar lavage fluid (BAL) of young patients with CF were found even in the absence of positive bacterial cultures [6–8]. BAL from infected children with other chronic respiratory conditions [9]. The altered production of inflammatory mediators was observed independently of the type of infectious stimulus, which suggests that the inflammatory response to bacterial infection is exaggerated in the airways of patients with CF [9].

The reason for the enhanced inflammatory response to pathogens and persistent neutrophil infiltrates into the airways is uncertain. It has been proposed that the production of proinflammatory mediators by airway epithelial cells may be altered in individuals with CF [10–14]. Another possibility is that chronic bacterial infection, combined with decreased clearance of bacterial breakdown products or exoproducts as a result of altered secretions, may result in the continued presence in the airways of proinflammatory stimuli [9].

Various functions have been ascribed to chemokines, including proinflammatory activities mediated by chemotaxis, chemokinesis, integrin activation, adhesion, and degradation of distinct leukocyte subsets expressing different chemokine receptors [15, 16]. In addition to IL-8, several chemotactic factors have been identified in BAL from patients with CF, including bacterial chemotactic peptides (FMLP [formylmethionylleucylphenylalanine]), products of the complement cascade (C5a), secreted products of stimulated phospholipid metabolism (platelet-activating factor [PAF] and leukotriene B4 [LTB4]), and degradation products derived from the extracellular matrix protein elastin or from the complex between elastase and its endogenous inhibitor, α1-antiprotease [17–21]. The relative importance of these factors, however, is not known, nor is it known which factors are present simultaneously in bronchial secretions. Considering the pivotal roles of chemoattractants in the inflammatory response, the identification of new chemo-
tactic factors and the characterization of their mechanisms of action are much needed to improve understanding of the pathogenesis of CF.

In a recent study, we determined the chemotactic activity of bronchial secretions from patients with CF before and after antimicrobial treatment. We separated, by gel filtration of these bronchial secretions, a 0.5–1-kDa fraction (“pool C”) with chemotactic activity higher than that of IL-8 [22]. To further characterize the bioactivity of pool C, we studied its biological activity and analyzed its response to a variety of physical treatments. We have also semipurified the chemotactic factor present in pool C by Sep-Pak (Waters) fractionation and determined the signal transduction mechanisms that semipurified chemotactic factor (SCF) activates on neutrophils. The chemotactic factor evoked chemotaxis and chemokinesis of neutrophils in the absence of superoxide production or release of primary granules. This suggests that SCF is a novel chemoattractant that directs neutrophil movement toward the airway epithelium.

Materials and Methods

Bronchial secretions. Bronchial secretions were collected between 1992 and 1994 from 8 patients with CF (age range, 12-46 years) hospitalized at the Geneva University Hospital (Geneva). The diagnosis of CF was established by clinical features and was confirmed by a sweat test. All patients had moderate-to-severe lung disease, and the mean (±SEM) forced expiratory volume in 1 s (FEV1; given as a percentage of the predicted value) was 41% ± 3%. The mean (±SEM) white blood cell count was 11,923 ± 1162 cells/mm³. All patients were colonized with Pseudomonas aeruginosa and were treated with the combination of an aminoglycoside and a cephalosporin. This treatment was associated with improved FEV1 (51% ± 4%) and lower white blood cell counts (6979 ± 210 cells/mm³). The patients were in stable condition and had no exacerbation of respiratory disease. Bronchial secretions collected during a 24-h period were pooled. The secretions were mixed with an equal volume of sterile 0.9% NaCl. The mixture was centrifuged, and supernatant was stored at -70°C until assays were done. The chemotactic factor recovered from the bronchial secretions of 3 patients was semipurified; 2 of those patients were homozygous for the ΔF508 mutation of CFTF.

Gel-filtration chromatography. For gel-filtration chromatography, processed bronchial secretions were heated at 95°C for 15 min, centrifuged, and passed through Millex filters with 0.22-mm pores (Millipore). Filtered samples were subjected to fractionation on a 1 × 120-cm Sephadex G75 gel chromatography column (Pharmacia). The column was eluted with PBS at 4 mL/h, and 1-mL fractions were collected. Three peaks (A, B, and C) showed chemotactic activity: peak A corresponded to IL-8, peak B to FMLP-like peptides, and peak C to a yet-identified factor [22]. In contrast to peak A (IL-8), peak C was detected in all patients, although to varying degrees. IL-8 content and peak C activity were not affected by antibiotic treatment, which indicates that no relationship existed between infection and peak C activity [22]. In 3 patients, peak C fractions were pooled (pool C) for further assays and purification.

Chemotaxis and chemokinesis. Chemotactic activity was determined by a modified Boyden chamber assay, as described elsewhere [22]. Polymorphonuclear neutrophils from buffy coats of citrated blood collected from healthy donors were isolated by dextran sedimentation, followed by density-gradient centrifugation in Ficoll-Paque (Amersham), according to standard procedure. Neutrophils were quantified by a colorimetric determination of the cleavage of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Boehringer Mannheim). Results were expressed as chemotactic index (CI), which was defined as the ratio between the total number of migrated neutrophils and the number of neutrophils that migrated nonspecifically. In some experiments, neutrophils were preincubated for 10 min at 37°C with 500 μM BocMLP (Sigma), an FMLP antagonist; 1 μM Ly293111 (Lilly Research), an LTB4 antagonist; or 10 μM CV-6209 (Biomol), a PAF antagonist, before the chemotactic assay. All assays included 10 nM FMLP (Sigma), 100 nM LTB4 (Biomol), and 1 μM PAF (Bachem) as positive controls. To test the signal transduction pathway, neutrophils were incubated for 30 min at 37°C with pertussis toxin (PTX; 0.5–1 μg/mL), an inhibitor of Gq/G11GTP-binding protein, or thapsigargin (0.01–1 μM), a Ca²⁺-ATPase inhibitor.

CIs were also measured for various concentrations of chloride. Without modification, the incubation medium for neutrophils, referred to as “normal medium,” contains 130 mM chloride (isotonic). Media were prepared in which the chloride concentration was decreased to 115 mM (hypotonic) or increased to 170 mM (hypertonic). To test whether osmolarity itself was a significant influence, a medium in which the extra chloride was replaced with glycerol was prepared by addition of 110 mM glycerol (Sigma) to the low-chloride solution. This procedure produced osmolarity the same as that of the high-chloride medium.

To distinguish chemotaxis from chemokinesis, chemotactic assays were performed with different amounts of pool C, either in the upper and lower compartments or in the lower compartment only of a Boyden chamber. The results were collected in checkerboard form and used to calculate chemotaxis (change in the directional response to the stimulus) and chemokinesis (change in the extent of the random locomotion), as described in [23].

Analysis of the physical characteristics of the chemical factors. The stability of the chemotactic factors was tested by evaluation of the chemotactic activity of pool C in response to different chemical and physical treatments. Results are expressed as a percentage of the result obtained for the corresponding control. Pool C was treated for 1 h at 37°C with trypsin (40 μg/mL), chymotrypsin (40 μg/mL), or proteinase K (50 μg/mL). At the end of the incubation period, enzymatic activity was stopped by heating of the samples at 95°C for 15 min. To determine the stability of pool C in response to changes in temperature, the samples were heated (95°C for 15 min) and subjected to 3 freeze (liquid nitrogen)/thaw (37°C) cycles. Bioactivity was also measured after samples were vacuum dried using a SpeedVac (Savant) at room temperature for 24 h. To measure the stability of pool C in response to changes in pH, samples were exposed to 1/1000 trifuoroacetic acid (pH 2) and 1.4 N NaOH (pH >10) for 1 h at room temperature and vacuum dried (SpeedVac).

Sep-Pak fractionation. The chemotactic factor present in pool C was purified further by reverse-phase chromatography using Sep-Pak C18 cartridges. Pool C was first acidified with 1/1000 trifluoroacetic acid to allow maximum binding on the cartridge. Fra-
Table 1. Effects of FMLP, leukotreine B4 (LTB4), and platelet-activating factor (PAF) antagonists on the chemotactic activity of pool C.

<table>
<thead>
<tr>
<th>Chemoattractant</th>
<th>No. of experiments</th>
<th>Response to indicated antagonist, mean ± SEM, chemotactic index</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Pool C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>15</td>
<td>15.4 ± 0.4</td>
</tr>
<tr>
<td>Patient 2</td>
<td>8</td>
<td>17.1 ± 0.5</td>
</tr>
<tr>
<td>Patient 3</td>
<td>10</td>
<td>14.8 ± 0.35</td>
</tr>
<tr>
<td>FMLP</td>
<td>4</td>
<td>11.6 ± 1.65</td>
</tr>
<tr>
<td>LTB4</td>
<td>4</td>
<td>11.2 ± 1.7</td>
</tr>
<tr>
<td>PAF</td>
<td>4</td>
<td>6 ± 0.8</td>
</tr>
</tbody>
</table>

NOTE. Pool C from 3 different patients with cystic fibrosis was tested for chemotactic activity in the absence (control) or presence of antagonists for FMLP (BocMLP, 500 μM), LTB4 (Ly293111, 1 μM), and PAF (CV-6209, 10 μM). In parallel experiments, the inhibiting effects of these antagonists on FMLP, LTB4, and PAF, respectively, were also tested. ND, not determined.

* a, vs. control.

Figure 1. Bioactivity of the chemotactic factor in pool C after exposure to a variety of physical treatments. A, The chemotactic index (expressed as a percentage of untreated samples) of pool C resisted protease digestion by trypsin (Try), chymotrypsin (C-Try), and proteinase K (PK). B, Pool C bioactivity was not affected by heating from room temperature (RT) to 95°C, freeze/thaw (F/T) cycles, or vacuum drying (V/D). C, Pool C bioactivity was not altered at extreme basic pH but showed a modest decrease at pH 2. *P < .05. Values are mean ± SEM of 4–6 experiments.
Trifugation, the supernatant was assayed for myeloperoxidase with the content of the myeloperoxidase could be determined. After centrifugation of a Boyden chamber after the addition of pool C to the lower compartment (solid line). Chemotaxis was measured in a similar manner, except that doses of pool C were added to both compartments of the chamber (dotted lines). As shown, the chemotactic index increased with larger doses of pool C, indicating that the chemoattractant exhibits chemotaxis and chemokinesis. Values are mean ± SEM of 3 experiments.

Myeloperoxidase assay: Myeloperoxidase in relation to total myeloperoxidase activity. The cells were pretreated with 5 μg/mL cytochalasin B for 15 min at 37°C before degranulation was initiated. The degranulation assay was performed at 37°C for 30 min in the absence or presence of SCF (72×), 1 μM FMLP as a positive control, or 0.1% Triton X-100. Triton X-100 was used to lyse the cells so that the total granule content of the myeloperoxidase could be determined. After centrifugation, the supernatant was assayed for myeloperoxidase with 2,2'-azino-bis-(3-ethylbenzthiozoline-6)-sulfonic acid as substrate in 100 mM citrate buffer (pH 4.2). The optical density was measured at 405 nm with a 3500-UV Bio-Rad ELISA reader.

Superoxide production assay: The release of superoxide anion by neutrophils was measured by the reduction of cytochrome c (Sigma) in a kinetic spectrophotometric microplate assay, using a Bio-Rad reader. The assay was performed at 37°C and included the buffer as a negative control, 1 μM FMLP as a positive control, or SCF (74×). The samples were preheated at 37°C for 15 min before the reaction was started by the addition of neutrophils and cytochrome c in excess. The optical density was read at 550 nm (narrow-bandwidth optical filter) over a period of 2 min. The reduction of cytochrome c, which is an indicator of superoxide production, followed an exponential curve as a function of time. Time constants (OD/min) were determined in the linear part of the curve.

Statistical analysis: Myeloperoxidase assay data were compared using Student’s t test. Values are expressed as the mean ± SEM of an individual experiment done in triplicate and repeated n times. P < .05 was considered to be significant.

Results

Failure of inhibition of FMLP, LTB4, and PAF receptors to affect neutrophil migration stimulated by pool C: Sputum samples from patients with CF were found to be chemotactically active. The separation by gel filtration of bronchial secretions from these patients revealed a 0.5–1-kDa fraction (pool C) with chemotactic activity that is distinct and greater than that of IL-8 [22]. The chemotactic activity of pool C from 3 patients was further examined. As shown in table 1, the CI of pool C was not affected by the presence of antagonists for FMLP, LTB4, or PAF. In contrast, the chemotactic activity induced by FMLP, LTB4, and PAF was inhibited in the presence of their respective antagonists (table 1). These results indicate that the molecular identity of the chemotactic factor in pool C is distinct from FMLP, LTB4, and PAF.

Physical characteristics of pool C bioactivity: As shown in figure 1, the bioactivity of pool C appears to be stable after application of a variety of physical treatments. It is not significantly inactivated by proteases, such as trypsin, chymotrypsin, or proteinase K (figure 1A). The chemotactic activity of pool C is not abolished after boiling, repeated freeze/thaw cycles, or vacuum drying (figure 1B). A small but significant decrease in CI was observed in samples of pool C exposed to an acidic pH, whereas the bioactivity of pool C remained stable after exposure to a basic pH (figure 1C).

Direct signaling of neutrophils by pool C: We next sought
to determine the effects of pool C bioactivity on neutrophil recruitment. For these experiments, the CI was measured in the lower compartment of a Boyden chamber containing increasing doses of pool C; neutrophils were transferred to the upper compartment. Neutrophils migrated from the upper to the lower compartment as the chemotactic gradient of pool C increased (figure 2). In a second assay, pool C stimulated the migration of neutrophils from the upper to the lower compartment in the absence of a concentration gradient (figure 2). These results indicate that pool C induces chemotaxis and chemokinesis.

We next studied the effects of uncouplers of signal transduction pathways on neutrophils exposed to pool C. PTX is a specific inhibitor of Gαi GTP-binding proteins [25]. As shown in figure 3A, increasing concentrations of PTX diminished the CI for neutrophils exposed to pool C as well as to FMLP. Thapsigargin is a specific inhibitor of the endoplasmic reticulum Ca2+-ATPase [26], which induces the depletion of internal Ca2+ stores. Thapsigargin markedly decreased the chemotactic response of neutrophils induced by pool C and FMLP (figure 3B). These data suggest that the factor in pool C directly stimulates chemotactic responses in neutrophils through G protein–coupled signal transduction pathways.

Biological activities of SCF. To further investigate the biological activities of the factor in pool C, the pooled samples were purified and concentrated using a Sep-Pak extraction procedure. The Sep-Pak columns were eluted with increasing concentration of acetonitrile. As shown in figure 4, the chemotactic activity of SCF was recovered in a fairly hydrophilic fraction of 40%-50% acetonitrile. This activity was not detected after extraction with chloroform (data not shown).

The chemotactic activity of pool C and SCF was determined for various concentrations of chloride and compared with that of FMLP. As shown in table 2, neutrophil chemotaxis in response to FMLP was not influenced by the chloride concentration. The bioactivity of the chemotactic factor was slightly increased when the NaCl concentration was raised from 115 to 170 mM. This effect appeared to be independent of the change in the osmolarity of the incubation medium, as evidenced by addition of glycerol to the low-chloride medium (table 2). However, the chemotactic factor bioactivity under isotonic conditions did not differ from that seen under hypertonic conditions.

To determine the ability of SCF to mobilize changes in the [Ca2+]i concentration after interaction with surface receptors, the [Ca2+]i concentration was monitored by digital imaging in neutrophils loaded with the Ca2+ probe Oregon-Green. As shown in figure 5, superfusion of SCF (8×) induced an increase in the [Ca2+]i concentration in most neutrophils. The time course of fluorescence changes exhibited a transient response, with a peak in [Ca2+]i that was reached after 10 s of stimulation and returned to baseline levels after 1 min. Slow oscillations of [Ca2+]i were also observed during the descending phase of the fluorescent signal. The amplitude of the [Ca2+]i increase induced by SCF was similar to that evoked by FMLP.

We next sought to investigate whether SCF could elicit the release of granules and the production of superoxide by neutrophils. As shown in figure 6, SCF (72×) failed to stimulate degranulation of neutrophil primary granules, in contrast to FMLP (figure 6A). Moreover, SCF (74×) did not stimulate the production of superoxide, in contrast to FMLP (figure 6B). Lower concentrations (40× and 20×) of SCF yield similar results (data not shown).

Discussion

Polymorphonuclear leukocytes respond to inflammatory mediators by migrating to sites of inflammation, where they may exert their cytotoxic activity. In CF, the migration of neutrophils into the airways is excessive, leading to a persistent inflammatory

**Table 2.** Effects of chloride concentration on the chemotactic activity of pool C and semipurified chemotactic factor (SCF).

<table>
<thead>
<tr>
<th>Chemoattractant</th>
<th>Response to indicated antagonist, mean ± SEM, chemotactic index (no. of measurements)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>115 mM NaCl</td>
</tr>
<tr>
<td>FMLP</td>
<td>6.6 ± 0.6 (8)</td>
</tr>
<tr>
<td>Pool C/SCF</td>
<td>3.3 ± 0.5 (3)</td>
</tr>
</tbody>
</table>

**NOTE.** Chemotactic activity was determined for FMLP (10 nM) and for SCF in the presence of increasing concentrations of NaCl (115-170 mM). The effect of osmolarity was evaluated by adding 110 mM of glycerol to medium containing 115 mM NaCl. Results for pool C and SCF were pooled. Data are from 3 experiments.
reaction and progressive destruction of the respiratory epithelium [1, 2]. Here we report the physical and biological characteristics of a novel chemoattractant for neutrophils that was semipurified from the bronchial secretions of patients with CF.

Fractionation by gel filtration of the bronchial secretions of patients with CF revealed a 0.5–1-kDa fraction (pool C) with chemotactic activity that remained stable in the presence of a variety of physical treatments. Pool C bioactivity was resistant to pH extremes, freeze/thaw, vacuum drying, boiling, and protease digestion. Because small peptides can be resistant to proteolysis, it is difficult to ascribe pool C bioactivity to a polypeptide, as opposed to another type of molecule. Pool C bioactivity could be recovered in 40%–50% acetonitrile, indicating that SCF is poorly hydrophobic. This was confirmed by extraction in chloroform, which failed to solubilize SCF bioactivity (data not shown), suggesting that the chemotactic factor in pool C is not a lipid. In addition, UV spectral characteristics of SCF differed from those of ATP or cAMP. The low molecular weight of SCF also clearly distinguished this factor from the well-known CXC chemoattractant IL-8 [27]. Although subsets of bacteria-derived n-formyl peptides may well be retained in pool C, the possibility that traces of FMLP or other low–molecular-weight chemoattractants, such as LTB4 and PAF, contributed was excluded by the use of specific antagonists that did not affect the bioactivity of pool C.

Human leukocyte chemoattractant receptors activate chemotactic and cytotoxic pathways to varying degrees. For example, FMLP, PAF, and LTB4 are potent chemoattractants for neutrophils that operate via the activation of PTX–sensitive G protein–coupled receptors. The binding of these chemoattractants to the corresponding cell-surface receptors also promotes mobilization of Ca2+ from intracellular stores, rearrangement of cytoskeletal elements, exocytosis, induction of surface receptors, adhesion, and activation of the respiratory burst system with release of superoxide anion via NADPH oxidase activation [28–30]. There is clear evidence that these biological responses are determined by expression of different PTX-sensitive and PTX-insensitive G proteins coupled to receptors for chemoattractants [31].

In this context, SCF appears to share properties with classic chemoattractants for neutrophils. Thus, the chemotactic activity of pool C was abolished in the presence of PTX or thap-
trophicls, an event that correlates with epithelial dysfunction and thus with clinical symptoms. It is now clear that bacteria, inflammatory cells, and epithelial cells themselves may emit signals that regulate neutrophil movement and responses at such surfaces. In CF, loss of CF transmembrane conductance regulator chloride-channel function by airway epithelial cells results in abnormal transepithelial electrolyte and fluid transport [1, 2]. The chloride concentration is an important factor in lung defense, modulating antimicrobial activity and neutrophil function [3, 5, 13, 36]. SCF-induced bioactivity was maintained, regardless of whether conditions were hypo-, iso-, or hypertonic, which suggests that chloride concentration does not interfere with the migration of neutrophils to sites of inflammation.

Among other treatments, the aggressive use of antibiotic therapy has been largely responsible for the increased life span of patients with CF, although efficacy is limited by the emergence of antibiotic-resistant pathogens. In a previous study, antibiotic treatment of patients with CF with aminoglycosides and cephalosporins did not result in a decrease in the chemotactic activity of pool C obtained from their bronchial secretions [22]. Although bacterial pathogens in the airways of patients with CF are rarely eradicated, this observation may be an indication that origin of SCF is nonbacterial. It is worth mentioning that the chemotactic activity of pool C or SCF could not be recovered in vitro from human neutrophils or from a human CF airway epithelial cell line (M.C. and T.S.D., unpublished results). Other studies aimed at evaluating the anti-inflammatory effects of steroids and nonsteroidal drugs in patients with CF reported adverse effects and increased risks of dissemination of infection with panresistant bacteria [37–39]. In this context, the identification of inflammatory mediators is of importance in designing therapeutic interventions aimed at modulating the inflammatory response and, thereby, the severity of airway-wall damage. Thus, the possibility of developing novel types of anti-inflammatory agents by targeting CF-specific chemotactic factors may await more-thorough clarification of the in vivo biological functions of chemoattractants.

In summary, this study showed that bronchial secretions from patients with CF can be used as a tool to investigate some of the chemoattractants involved in leukocyte recruitment. The detection of SCF, a novel chemotactic factor for neutrophils, provides evidence of its involvement in the pathogenesis of CF. Although the molecular identification of SCF and its localization in the airways of individuals with and without CF wait further purification from larger quantities, it is possible that interference with the SCF-based signaling pathway may provide a potentially important new therapeutic target for approaches aimed at lowering chemotactic activity in bronchial secretions and controlling inflammation and tissue destruction.

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References


