Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation*

Wei Xiao, MD; Yao-Pi Hsu, MS; Akitoshi Ishizaka, MD; Teruo Kirikae, MD, PhD; and Richard B. Moss, MD

Background: Interest in airways inflammatory disease has increasingly focused on innate immunity. We investigated several components of innate immunity in induced sputum of patients with cystic fibrosis (CF), COPD, and asthma, and healthy control subjects.

Methods: Twenty eight patients with mild CF lung disease (age ≥ 12 years; FEV₁, 74 ± 3% predicted [mean ± SE]), 74 adults with COPD (FEV₁, 55 ± 2% of predicted), 34 adults with persistent asthma (FEV₁, 66 ± 2% of predicted), and 44 adult control subjects (FEV₁, 85 ± 1% of predicted) were studied while in stable clinical condition. Levels of sputum interleukin (IL)-8, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, human cationic antimicrobial protein 18 (CAP18), urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), and plasminogen activator inhibitor (PAI)-1 were determined. Cell sources were investigated by flow cytometry and immunohistochemistry. Spirometry was performed prior to sputum induction.

Results: CF patient sputum showed greatest increase in IL-8 compared to that of patients with COPD and asthma (which were also greater than control subjects), and elevated levels of TNF-α and IL-10 compared to other groups. There were no differences in IFN-γ. CAP18 levels were elevated in CF and COPD patients compared to control subjects, while asthma patients had reduced CAP18 levels. uPA levels were similar but uPAR was elevated in CF and COPD patients more so than in asthma patients, while PAI-1 levels were elevated in all three disease groups. CAP18 localized to neutrophil secondary granules; neutrophils were also sources of IL-8 and PAI-1. CAP18 and PAI-1 negatively correlated with pulmonary function.

Conclusion: Induced-sputum innate immune factor levels discriminate inflammatory changes in CF, COPD, and asthma, suggesting potential roles in pathophysiology and as well as providing disease-specific biomarker patterns.

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Key words: cathelicidin; cystic fibrosis; cytokine; innate immunity; urokinase plasminogen activator system

Abbreviations: BSA = bovine serum antigen; CAP18 = human cationic antimicrobial protein 18; CF = cystic fibrosis; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; IFN = interferon; IL = interleukin; PAI = plasminogen activator inhibitor; PBS = phosphate-buffered saline solution; PE = phycoerythrin; TNF = tumor necrosis factor; uPA = urokinase-type plasminogen activator; uPAR = uPA receptor

The pathogenesis of cystic fibrosis (CF) lung disease is characterized by compromised local innate immunity, which permits microbial colonization and chronic infection.¹ Current thinking emphasizes the primary role of volume depletion of airway surface liquid and resulting compromise of mucociliary clearance.² Additional innate defense mechanisms may also be involved, as a primary proinflam-

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matory bias of the CF epithelia has been posited, and activity of endogenous antimicrobial peptides produced by airway epithelia and glands may be altered in CF. While some epithelial antimicrobials have received much attention in CF, in particular the β-defensins, others have not. In particular, the role of the only human cathelicidin, the 18-kd, 140 amino acid cationic antimicrobial protein (human cationic antimicrobial protein 18 [CAP18]), has not been investigated in sputum from patients with CF. It is produced by respiratory epithelia as well as stored in secondary (specific) granules of polymorphonuclear leukocytes, and in a model system restores deficient antimicrobial activity in the CF airway milieu. Proteolytic cleavage of CAP18 by proteinase 3 yields a potent antimicrobial peptide (carboxy terminal 37 amino acid fragment of CAP18). Via its action on the formyl peptide receptor-like 1 expressed on several cell types, carboxy terminal 37 amino acid fragment of CAP18 is also an important regulator of macrophage function; has potent chemotactic activity for neutrophils, monocytes, and T cells; and possesses angiogenesis activity. Abnormalities in CAP18 could therefore profoundly affect the pathophysiology of CF by its ability to link innate to adaptive immunity and its neovascularizing effect.

Another innate defense pathway recently found active in the airway is the plasminogen activator system constituted by its local serine protease activator urokinase-type plasminogen activator (uPA), the uPA-specific cell surface receptor (uPAR receptor [uPAR]) [CD87], and an arginine-specific serine protease inhibitor (serpin), plasminogen activator inhibitor (PAI)-1. Via its action on the formyl peptide receptor-like 1 expressed on several cell types, carboxy terminal 37 amino acid fragment of CAP18 is also an important regulator of macrophage function; has potent chemotactic activity for neutrophils, monocytes, and T cells; and possesses angiogenesis activity. Abnormalities in CAP18 could therefore profoundly affect the pathophysiology of CF by its ability to link innate to adaptive immunity and its neovascularizing effect.

Materials and Methods

Subjects
We recruited 28 patients with CF (age, 12 to 50 years) followed up at the CF Center clinic at Stanford University Medical Center. CF diagnoses in all patients were made by positive (>60 mEq/L) pilocarpine iontophoresis sweat test results, with homozygous or compound heterozygous for ΔF508 CF transmembrane conductance regulator mutations. All patients had chronic infection with Pseudomonas aeruginosa by serial sputum culture and were in stable clinical condition (no pulmonary exacerbation within previous month). CF patients were also excluded for FEV1 values <40% predicted, oxyhemoglobin saturation <92% on room air, pneumothorax, hemothysis, or history of Burkholderia cepacia in sputum. None were receiving regular inhaled or systemic corticosteroids. Seventy-four patients (age 38 to 79 years) with previously diagnosed COPD were recruited from the Respiratory Clinic of the Hospital of Shandong Medical University, Peoples Republic of China. All were in stable condition and not receiving antibiotics for at least 2 weeks prior to testing. Thirty-four patients with asthma (age, 14 to 75 years) followed up in Shandong were also studied. Finally 44 healthy nonsmoking subjects without reported respiratory symptoms (age, 20 to 60 years; 20 patients at Stanford and 24 patients at Shandong) were also studied. All participants gave written informed consent with protocols approved by the Institutional Review Boards at Stanford and Shandong.

Pulmonary Function

Pulmonary function tests were performed according to American Thoracic Society guidelines for performance and acceptance prior to sputum induction.
Sputum Induction and Processing

Sputum was collected from each patient as previously described using 3% hypertonic saline solution (at Stanford) or 3.5% hypertonic saline solution (at Shandong) via an ultrasonic nebulizer with 2-min collections of sputum, which were pooled for analysis. All subjects underwent sputum induction regardless of history of ability to expectorate. Subjects were encouraged to cough, and sputum was collected into polypropylene cups. The induced-sputum samples were weighed, and an equal volume of Sputolyisin (Calbiochem-Novabiochem; San Diego CA) diluted 10% in normal saline solution was added. Samples were vortexed 3 seconds and incubated for 5 min at 37°C in a water bath with vigorous shaking (160 rotations per minute). Samples were further mixed by aspirating up and down 20 times in a transfer pipette. Five-minute incubations were then repeated two more times. Finally, the samples were centrifuged at 2,000 revolutions per minute (800g) for 5 min at 4°C, and the sol phase was used for analysis.

Soluble Mediators of Innate Immunity

Human IL-8, IL-10, IFN-γ, TNF-α, CAP18, uPA, uPAR, and PAI-1 levels in the supernatant sol phase of sputum were determined by enzyme-linked immunosorbent assay (ELISA) using a standardized format. Wells of microtiter plates (polystyrene 96-well culture clusters, Catalog No. 3598; Costar; Pleasanton CA) were coated with 50 μL per well capture antibody (see below for specific reagents) diluted in phosphate-buffered saline solution (PBS) [P-4417; Sigma Chemical; St. Louis, MO], incubated overnight at 4°C, and washed three times with wash buffer (PBS 0.01%, Thimerosal; Sigma Chemicals; 0.05% Tween 20 [polyoxyethylene sorbitan mono-oleate]; Sigma Chemical). Blocking solution (1% bovine serum albumin [BSA], A-2153; Sigma Chemical; 5% sucrose PBS) 200 μL per well was added, incubated at room temperature for 1 h, and the wells were washed three times with washing buffer. Samples and standards diluted in diluting solution (0.1% BSA-0.05% Tween 20-Tris-buffered saline solution) were then added (50 μL per well), incubated overnight at 4°C, and washed three times with washing buffer. Biotinylated detection antibody (see below for specific reagents) in dilution buffer was then added (50 μL per well) and incubated 2 h at room temperature with gentle mixing. Plates were then washed four times with washing buffer. Avidin-peroxidase-conjugated secondary antibody (see below for specific reagent) diluted in dilution buffer was then added (50 μL per well), and incubated 1 h at room temperature with gentle mixing. Plates were then washed four times with washing buffer. Developing solution (75 μL per well α-phenylenediamine [P-6912; Sigma Chemical] in citrate-phosphate buffer pH 6.0 with 4 μL 3% H₂O₂ [H-1009; Sigma Chemical]) per 10-mL buffer was added, the reaction stopped with 25 μL per well 2 N H₂SO₄, and the well color was read at an optical density of 492 with an automated microplate reader (Molecular Devices; Mountain View, CA).

Specific Reagents for the ELISA

IL-10: For IL-10, the capture antibody was from Pharmingen (Catalog No. 18551D; BD Pharmingen; San Diego, CA), the primary antibody was purified rat anti-human IL-10 diluted to 4 μg/mL, the detection antibody was biotinylated rat anti-human IL-10 (Catalog No. 18562D; BD Pharmingen) diluted to 4 μg/mL, the detector was horseradish peroxidase-streptavidin (Catalog No. 43–4323; Zymed Laboratories; San Francisco, CA) diluted 1:1000, and the standard was recombinant human IL-10 (Catalog No. 19701N; BD Pharmingen) diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IFN-γ: For IFN-γ, the capture antibody was purified mouse anti-human INF-γ (NIB4, Catalog No. 18991D; BD Pharmingen) diluted to 2 μg/mL, the detection antibody was biotinylated mouse anti-human IFN-γ (4S13, Catalog No. 18902D; BD Pharmingen) diluted to 2 μg/mL, and the standard was recombinant human IFN-γ (Catalog No. 19751N; BD Pharmingen), diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IL-8: For IL-8, the capture antibody was purified monoclonal antibody to human IL-8 (Catalog No. MAB208; R&D Systems; Minneapolis, MN) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human IL-8 (Catalog No. BAF208; R&D Systems) diluted to 200 μg/mL, and the standard was recombinant human IL-8 (Catalog No. 208-IL; R&D Systems) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, and 75 pg/mL.

TNF-α: For TNF-α, the capture antibody was mouse anti-human TNF-α (Part 840119; R&D Systems) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human TNF-α (Part 840120; R&D Systems) diluted to 300 ng/mL, the detector was horseradish peroxidase-streptavidin (Part 89080; R&D Systems) diluted to 1:200, and the standard was recombinant human TNF-α (Part 840121; R&D Systems) diluted to 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 16 pg/mL.

uPA, PAI-1, and uPAR: For uPA, PAI-1, and uPAR, ELISA kits (Imubind; American Diagnostics; Greenwich, CT) were used according to the instructions of the manufacturer (Catalog Nos. 894, 821, and 893, respectively).

CAP18: For CAP18, the capture antibody was rabbit polyclonal antibody to human lipopolysaccharide-binding domain of CAP18 diluted to 1:200; the detection antibody was a mouse IgG monoclonal antibody to CAP18 diluted to 1:1000; the detector was horseradish peroxidase-conjugated goat anti-mouse IgG (Catalog No. 115–367-3296; Jackson Immuno Research Lab Inc.; West Grove, PA) diluted 1:2500; and the standard was a synthetic 27 amino acid peptide fragment of CAP18 (amino acids 109–135) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, 78, 39, and 20 ng/mL. The capture antibody, detection antibody and standard were obtained from Dr. Yoshikazu Naiki and Tenuo Kirikae, Japan. In a pilot study, the same CAP18 ELISA was used to measure CAP18 levels in serum, BAL fluid, and expectorated sputum of patients with CF (see “Results”).

Immunohistochemistry of Induced-Sputum Cells

Adherence of Sputum Cells to Glass Slides: After removal of the supernatant of centrifuged sputum samples, cell pellets were each suspended in 20 mL of PBS (P-4417; Sigma Chemical) and centrifuged at 1,200 revolutions per min for 10 min. After removal of the supernatant, the cell pellet was resuspended in 1% BSA (A-2153; Sigma Chemical)-PBS. Dead cells were excluded by Trypan Blue (T-920; Sigma Chemical) cell count. Cells were diluted to approximately 2.5 × 10³ in 1% BSA-PBS. Following cytocentrifuge (700 revolutions per minute for 5 min) 100 μL of cell suspension per slide were air dried and freezer stored at −20°C.

Staining: Frozen slides were thawed at room temperature. Cells were fixed by incubating slides in 4% formaldehyde (Catalog No. 16220; Electron Microscopy Sciences; Fort Washington PA)-PBS for 15 min at room temperature to fix cells. After three washes in PBS, slides were incubated in 1% H₂O₂ (H-1009; Sigma Chemical)-PBS 10 min at room temperature, washed three times in PBS, and stained. Slides were permeabilized by incubation with 0.5% saponin (S-2149; Sigma Chemical)-PBS for 10 min at room temperature. Slides were then washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal (T-5125; Sigma). Once slides were washed three times in PBS, slides were incubated in 1% H₂O₂ for 10 min. After removal of the peroxidase reaction stop solution, the same buffer was used after this step. According to the protocol of the Vectastain Elite ABC kit

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(Catalog No. PK-6102; Vector Laboratories; Burlingame, CA), slides were incubated for 20 min at room temperature in blocking solution (5% horse serum-PBS for uPAR, PAI-1, CAP18, IL-8, CD14, and cytokeratin; 5% goat serum for CD15) and washed thrice with PBS. Slides were then incubated in avidin blocking solution (Catalog No. SP-2001; Avidin/Biotin Blocking Kit; Vector Laboratories) for 15 min at room temperature, washed thrice with PBS for surface staining, and then washed in PBS-0.05% Tween 20–0.01% Thimerosal for 5 min. Slides were then incubated with biotin blocking solution for 15 min at room temperature and washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal. Following this preparation, slides were incubated with primary monoclonal antibody (for uPAR, Catalog No. 3936: American Diagnostica; Greenwich, CT; for PAI-1, Catalog No. 37575; American Diagnostica; for CAP18, see primary antibody; ELISA methods above; for IL-8, Catalog No. 534717; BD Pharmingen mouse IgG2b clone G265–8; for CD14, Catalog No. 555396; BD Pharmingen; and for cytokeratin, Catalog No. 349205; BD Pharmingen) each diluted in 5% horse serum-1% BSA-0.05% Tween 20–PBS. For CD15, the antibody (Catalog No. 555400; BD Pharmingen) was diluted in 5% normal goat serum-1%BSA-0.05% Tween 20–PBS. For CD15, the reaction was developed with biotinylated goat anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) for 15 min at room temperature, washed thrice with PBS-0.05% Tween 20–PBS. Slides were incubated with primary antibody for 60 min at room temperature, and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. An isotype control antibody was run with each experiment (mouse IgG isotype control; Catalog No. I-2000; Vector Laboratories). For staining uPAR, PAI-1, CAP18, IL-8, CD14, and cytokeratin, reactions were developed with biotinylated horse anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% horse serum-1% BSA-0.05% Tween 20–PBS. For staining CD15, the reaction was developed with biotinylated goat anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% goat serum-1% BSA-0.05% Tween 20–PBS. Slides were incubated for 30 min at room temperature and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. For development, slides were incubated for 30 min at room temperature with Vector ABC reagent (Catalog No. PK-6102; Vector Laboratories) prepared in PBS 30 min before use and then washed thrice in PBS. Color was developed (3,3′-diaminobenzidine peroxidase substrate; Catalog No. SK-4100; Vector Laboratories) at room temperature and washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal for cytoplasmic staining. Slides were then incubated with biotin blocking solution for 15 min at room temperature and washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal. Following this preparation, slides were incubated with primary monoclonal antibody (for uPAR, Catalog No. 3936: American Diagnostica; Greenwich, CT; for PAI-1, Catalog No. 37575; American Diagnostica; for CAP18, see primary antibody; ELISA methods above; for IL-8, Catalog No. 534717; BD Pharmingen mouse IgG2b clone G265–8; for CD14, Catalog No. 555396; BD Pharmingen; and for cytokeratin, Catalog No. 349205; BD Pharmingen) each diluted in 5% horse serum-1%BSA-0.05% Tween 20–PBS. For CD15, the antibody (Catalog No. 555400; BD Pharmingen) was diluted in 5% normal goat serum-1%BSA-0.05% Tween 20–PBS. Slides were incubated with primary antibody for 60 min at room temperature, and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. An isotype control antibody was run with each experiment (mouse IgG isotype control; Catalog No. I-2000; Vector Laboratories). For staining uPAR, PAI-1, CAP18, IL-8, CD14, and cytokeratin, reactions were developed with biotinylated horse anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% horse serum-1% BSA-0.05% Tween 20–PBS. For staining CD15, the reaction was developed with biotinylated goat anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% goat serum-1% BSA-0.05% Tween 20–PBS. Slides were incubated for 30 min at room temperature and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. For development, slides were incubated for 30 min at room temperature with Vector ABC reagent (Catalog No. PK-6102;Vector Laboratories) prepared in PBS 30 min before use and then washed thrice in PBS. Color was developed (3,3′-diaminobenzidine peroxidase substrate; Catalog No. SK-4100; Vector Laboratories) according to manufacturer’s instruction. Color development was stopped by washing in distilled water, the slides were air dried, and the slides were viewed under microscope to assess the positive staining cells and determine cell type by comparing with Wright-Giemsa-stained slides (Hema 3 set, Catalog No. 122–911; Biochemical Science; Swedesboro, NJ).

Flow Cytometry

Five × 10⁶ Sputolysin-treated (Calbiochem Corporation; San Diego, CA), PBS-washed sputum cells were added to each assay tube, incubated in 5% BSA-PBS for 10 min at room temperature to block nonspecific binding, washed once in PBS, fixed in 4% formaldehyde-PBS for 15 min at room temperature, and washed once with PBS. The cell samples were permeabilized in 0.5% saponin-PBS for 10 min at room temperature and washed once in 0.1% saponin-0.5% BSA-PBS. Monoclonal anti-human IL-8, uPAR, PAI-1, and CAP18 antibodies, and mouse IgG1, IgG2a, and IgG2b isotype control antibodies were labeled with fluorescein isothiocyanate (FITC) according to the zenon complex formation protocol (Zenon Alexa Fluor 488 mouse IgG1, IgG2a and IgG2b labeling kits, Catalog Nos. Z-25002, Z-25202, and Z-250102; Molecular Probes; Eugene, OR). Phycoerythrin (PE) labeled anti-human CD14, CD15, and cytokeratin were obtained from BD Pharmingen (Catalog Nos. 555398, 555402, and 347204, respectively). FITC- and PE-labeled anti-human antibodies (IL-

Results

The CF patients (17 women and 11 men) were generally young adults (mean ± SD age, 23.7 ± 11.1 years) with well-preserved pulmonary function (mean ± SD FEV₁, 74.0 ± 17.4% predicted). The patients with COPD (24 women and 50 men) were, as expected, older (59.2 ± 9.9 years) with generally greater airflow obstruction (FEV₁, 54.6 ± 13.7% predicted). The patients with asthma (19 women and 15 men) were intermediate between these other groups in age (47.4 ± 13.9 years) and airflow obstruction (FEV₁, 65.9 ± 13.0% predicted). All but two patients with asthma were receiving regular bronchodilator medication (β₂-adrenergic aerosols and/or theophylline), while only seven patients were receiving regular inhaled corticosteroids.

CAP18 in Serum, BAL Fluid, and Sputum

To study the biology of CAP18 in CF, in a pilot study we first measured serum CAP18 levels by ELISA in patients with CF in stable condition (n = 15) and compared these to samples obtained from CF patients on admission to hospital for treatment of pulmonary exacerbation (n = 15) and 15 healthy adult control subjects. The ELISA showed linear parallelism in the range of 3 to 3,000 ng/mL with interassay coefficient of variation < 15%. Levels of CAP18 were similar in the three subject groups (stable CF, 966 ± 980 ng/mL; exacerbation CF, 1,137 ± 685 ng/mL; and control, 1,012 ± 648 ng/mL). We next compared CAP18 levels in BAL fluid of 23 patients with CF and 12 control subjects. CF patients had significantly higher levels of BAL fluid CAP18 than control subjects (189.7 ± 18.7 ng/mL vs...
120.7 ± 24.7 ng/mL, p = 0.036, by two-sided unpaired t test). As this suggested increased local production and/or reduced metabolism within the pulmonary compartment of inflamed CF airways, we proceeded to measure CAP18 levels in expectorated sputum of 30 patients with CF. Expectorated CF sputum levels (177.4 ± 14.7 ng/mL) were quite similar those in BAL, suggesting that sputum is an easily accessible and representative sample of airway secretions for measurement of CAP18 levels.

In order to obtain sputum samples from CF patients with mild disease severity and little or no sputum productivity (including nonexpectorators), we then proceeded to study CAP18 levels in induced-sputum samples using a validated standardized methodology. All nonexpectorators were able to produce adequate sputum for analysis after induction. CF patients were compared to patients with COPD, patients with asthma, and healthy control subjects. CF and COPD patients had comparable levels (79.6 ± 93 ng/mL vs 75.3 ± 38.9 ng/mL, respectively) and significantly elevated levels of sputum CAP18 when compared to control subjects (39.9 ± 24.2 ng/mL, p < 0.009 for either group vs control), while asthmatics had significantly reduced IL-8 were significantly higher in patients with CF compared to patients with COPD or asthma, or control subjects (p < 0.05), without differences noted between the latter three groups. TNF-α levels showed a similar pattern as IL-10 with elevation in patients with CF compared to patients with COPD or asthma, or control subjects (p < 0.001). INF-γ levels were similar among all four groups.

uPA levels were similar among the four groups. In contrast, uPAR levels were elevated and similar in patients with CF and COPD compared to patients with asthma and control subjects (p < 0.001 for each); uPAR levels were also higher in asthma than controls (p < 0.05). PAI-1 levels were comparably elevated in patients with CF, COPD, and asthma, compared to control subjects (p < 0.05; Table 1).

**Neutrophil Origin of Sputum CAP18, IL-8, and PAI-1**

In order to examine the cellular sources of these sputum substances in patients with CF, we examined cells obtained from induced-sputum samples by immunohistochemical staining and independently by flow cytometry. Most sputum cells were either intact or more commonly degenerated polymorphonuclear neutrophils with variable mucus component and occasional other cell types such as monocytes, macrophages, eosinophils, or squamous epithelial cells; ciliated epithelial cells were not seen (Fig 1, top). CAP18 heavily stained the cytoplasmic granules of these neutrophils (Fig 1, center and bottom), suggesting that a major source of sputum CAP18 was infiltrating neutrophils. CAP18 is known to be localized to secondary or specific neutrophil granules. Using flow cytometry, CAP18 expression was markedly increased in airway cells that coexpressed CD15 (3-fucosyl-N-acetyl-lactosamine), which is present in the intracellular secondary granules of neutrophils.

### Table 1—Levels of Innate Immune Factors in Sputum of Patients With CF, COPD, and Asthma, and Healthy Control Subjects*

<table>
<thead>
<tr>
<th>Factors, pg/mL</th>
<th>CF (n = 28)</th>
<th>COPD (n = 74)</th>
<th>Asthma (n = 34)</th>
<th>Healthy (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP18</td>
<td>79,623 ± 18,597†</td>
<td>75,262 ± 4,583†</td>
<td>13,646 ± 1,678†</td>
<td>39,936 ± 3,644</td>
</tr>
<tr>
<td>IL-8</td>
<td>29,889 ± 4,226†</td>
<td>11,852 ± 1,880†</td>
<td>11,683 ± 3,707†</td>
<td>1,713 ± 317</td>
</tr>
<tr>
<td>IL-10</td>
<td>386 ± 87†</td>
<td>53 ± 14</td>
<td>36 ± 4</td>
<td>72 ± 14</td>
</tr>
<tr>
<td>INF-γ</td>
<td>794 ± 137</td>
<td>392 ± 65</td>
<td>627 ± 225</td>
<td>684 ± 117</td>
</tr>
<tr>
<td>TNF-α</td>
<td>74 ± 31†</td>
<td>10 ± 4</td>
<td>8 ± 2</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>uPA</td>
<td>59 ± 26</td>
<td>76 ± 24</td>
<td>64 ± 30</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>uPAR</td>
<td>803 ± 112†</td>
<td>583 ± 87†</td>
<td>390 ± 103†</td>
<td>85 ± 11</td>
</tr>
<tr>
<td>PAI-1</td>
<td>3,812 ± 469†</td>
<td>6,410 ± 1039†</td>
<td>4,802 ± 1127†</td>
<td>592 ± 97</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM. pg/mL.
†Significant at ≥ 95%. See text for p values.
confirming the importance of neutrophil specific granules as a major source of airway CAP18 in CF (Fig 2).

Another neutrophil product important in CF pathobiology, IL-8, was also identified by flow cytometry in sputum cells that expressed CD15 (Fig 3). Similarly, PAI-1 was found, albeit at lower levels of expression, in CD15+ sputum cells (Fig 4).

Sputum levels of innate immune factors were correlated with each other in order to examine potential relationships in production or regulation. As shown in Table 2, levels of the three plasmin activation system components correlated with each other (p < 0.0001) but also with several cytokine responses (eg, uPAR and uPA with IL-8, p < 0.0001; uPAR and uPA with TNF-α, p ≤ 0.002; and uPAR with IL-10, p = 0.006). In addition, uPAR was weakly correlated with CAP18 (p = 0.01). Among cytokines, IL-8 levels correlated with TNF-α and IL-10 (p < 0.0001), and IL-10 with IFN-γ (p = 0.001). CAP18 levels were correlated not only weakly with uPAR but also weakly with IL-8 (p = 0.04) and strongly with IL-10 (p < 0.0001).

To examine the potential role of these factors in disease activity or progression, innate immune factors were correlated with level of pulmonary function as determined by spirometric assessment of airflow obstruction (Table 2). Among all study subjects, FEV1 percentage of predicted showed a strong negative correlation with sputum CAP18 and PAI-1 levels (p ≤ 0.0009) and a weak negative correlation with uPAR levels (p = 0.01; Table 2). The overall negative correlation between pulmonary function and CAP18 was due primarily to results in patients with CF (r = −0.40, p = 0.06) and to a slightly lesser extent COPD (r = − 0.19, p = 0.11). A similar negative correlation trend was seen between FEV1 percentage of predicted and IL-8 in patients with CF (r = −0.39, p = 0.056) and patients with COPD (r = −0.21, p = 0.08). However, when innate factors were correlated with each other in patients with CF, CAP18 and IL-8 did not correlate with each other; instead, CAP18 levels correlated strongly with IL-10 (r = 0.78, p = 0.001) and IFN-γ (r = 0.59, p = 0.002), suggesting that elevated CAP18 levels in CF may represent a counterinflammatory response along with IL-10 and IFN-γ (IL-10 correlated strongly with IFN-γ, r = 0.58, p = 0.001). In contrast, levels of IL-8, a known proinflammatory factor in CF and COPD that we confirmed correlated negatively with pulmonary function (see above), correlated strongly with levels of uPAR (r = 0.59, p = 0.002), PAI-1 (r = 0.5, p = 0.009), and TNF-α (r = 0.5, p = 0.007). TNF-α correlated strongly with uPA (r = 0.65, p = 0.0004).

**Discussion**

Research1–2,30–31 has focused on the role of deregarded aspects of innate immunity in the pathobiol—
Figure 2. Flow cytometry for presence of CAP18 and CD15 in nonapoptotic (propidium iodide-negative) cells. Top: CF sample stained for CAP18 (purple) with isotype control antibody (open green) shown. Middle: same sample stained for CD15 with control. Bottom: two-color flow cytometry double-positive CAP18/CD15 cells are shown in upper right quadrant of gated cells (67.4% of total). Isotype control staining resulted in 1.0% double-positive cells (not shown). FL1 = fluorescence emissions wavelength 515 to 545 nm; FL2 = fluorescence emissions wavelength 564 to 606 nm.

Figure 3. Flow cytometry for presence of IL-8 and CD15. Top: CF sample stained for IL-8 with isotype control shown as for Figure 2. Middle: same sample stained with CD15 and control. Bottom: double-positive IL-8/CD15 cells shown in upper right quadrant (64.4% of total). See Figure 2 legend for expansion of abbreviations.
ogy of chronic inflammatory airways diseases such as CF, asthma, and COPD. In the present study, we were interested in aspects of the innate immune response in CF, in particular the following: (1) the role of the uPA system and the antimicrobial cathelicidin CAP18 that have not received much attention, and (2) how any abnormalities in CF compared to COPD and asthma as well as healthy persons. In order to study this local response noninvasively, we employed a standardized, validated sputum-induction protocol.29

The pulmonary plasminogen activator system involves multiple components including uPA, uPAR, and PAI-1. A saturable, specific binding of uPA to uPAR has been demonstrated in a number of cell types including monocytes, macrophages, mast cells, lymphocytes, fibroblasts, endothelial cells, and pulmonary airway epithelial cells.32–33 Mature uPAR lacks the hydrophobic transmembrane and intracellular domains. Instead, a glycosylphosphatidylinositol moiety is added to the C-terminus providing the anchorage to the outer leaflet of the plasma membrane. Soluble uPAR has been isolated from normal and diseased plasma. By interacting with cell surface adapter molecules and extracellular proteins, uPAR acts beyond the traditional role of localizing and activating cell surface uPA and extends its effects to cellular adhesion and signal transduction.32 The expression of uPAR is enhanced in various tumors and by a number of proinflammatory agents such as growth factors and cytokines.32–34 Intraperitoneal injection of endotoxin to mice increased the expression of uPAR in many tissues.35 Both membrane and soluble forms of uPAR were up-regulated in monocyte cultures by several bacterial surface proteins.34 There is thus reason to suspect that chronic airways infection, such as seen in CF and COPD, may up-regulate uPAR, and indeed we found increased

Figure 4. Flow cytometry for presence of PAI-1 and CD15. Top left: CF sample stained for PAI-1 with isotype control shown. Top right: same sample stained for CD15 and control. Bottom left: double-positive cells (upper right quadrant) representing 4.16% of total gated cells. Bottom right: double-positive cells in upper right quadrant (1% of total) using isotype control antibodies. See Figure 2 legend for expansion of abbreviations.
levels in sputum in CF and COPD, and to lesser degree in asthma, compared to normal. Interestingly, local uPA itself does not appear to be elevated in any of the disease groups, but its effects could be augmented by the up-regulated receptor levels seen.

PAI-1, a serine protease inhibitor belonging to the serpin family, is a single-chain 45- to 50-kd glycoprotein secreted by many cell types. It binds to uPA, activating the activity of uPA beyond plasmin activation to involve cell adhesion and tissue remodeling. PAI-1 may be inactivated by binding to uPA or forming complexes with uPA and uPAR that are internalized and digested in lysosomes. PAI-1 is secreted by many cell lines, and its expression can be regulated by hormones, growth factors, cytokines, and endotoxin in cell cultures. Depending on the presence of specific regulatory agents, the expression of PAI-1 can either be enhanced or reduced. We found comparably increased levels of PAI-1 in all three chronic inflammatory airways diseases studied (Table 3). Marshall and Shute reported quantitatively similar elevations of PAI-1 in CF sputum (mean, 5.7 ng/mL vs 1.0 ng/mL in control subjects, as compared with our mean of 3.8 ng/mL vs 0.6 ng/mL). During the inflammatory processes, autocrine and paracrine chemokine and cytokine secretion by neutrophils, monocytes, lymphocytes, and airway epithelial cells is enhanced. This may result in increased PAI-1 in sputum by different paths in these diseases.

Increases of the neutrophil chemotactic cytokine IL-8 level in airways of CF and COPD patients have been previously reported and related to pathogenesis. IL-8 is produced by a variety of cells including neutrophils, monocytes, T-cells, and endothelial and airway epithelial cells. IL-8 and uPAR both are chemotactic with monocyte and airway epithelial cell expression induced by bacterial products. The interaction between leukocytes and endothelial cells is regulated by IL-8 via changes in integrin expression that also involve uPAR. Therefore, integrins are potential mediators connecting the functions of IL-8 and uPAR (sputum levels of which we found to be highly correlated). Marshall et al have also related IL-8 activity to PAI-1 by demonstrating that PAI-1 enhances IL-8 activity via inhibition of shedding IL-8/heparan sulfate/syndecan-1 complexes from endothelium. Indeed, IL-8 levels were highly correlated in our study not only with uPAR but also with PAI-1, suggesting a coordinate response in these diseases. The plasminogen activator system may play a prominent role in sustaining airway inflammation by increasing β2-integrin-mediated leukocyte adhesion and also leukocyte adhesion to extracellular matrix vitronectin.

With regard to cathelicidin CAP18, we found comparably elevated levels in CF and COPD patients and subnormal levels in asthmatics. These differences were due to local production, as systemic CAP18 levels in CF patients and control subjects were similar to each other and previously reported plasma levels (approximately 1 μg/mL). CAP18 levels inversely correlated with pulmonary function, a relationship seen most strongly in CF patients but also in COPD. These changes have not been previously reported, although recently Chen et al found increased CAP18 in BAL fluid from patients with CF; CAP18 levels correlated with neutrophilia and decreased lung function. Under normal conditions, CAP18 is secreted by airway epithelial cells and alveolar macrophages, but in conditions of neutrophilia this cell type would be expected to be the dominant source, as we found. The antimicrobial characteristics of CAP18 are related to its α-helical

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**Table 2—Significant Correlations of Induced Sputum Innate Immune Factors With Each Other and With Pulmonary Function Among All Study Subjects**

<table>
<thead>
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<th>Factors</th>
<th>Correlates</th>
<th>r</th>
<th>p Value</th>
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<tr>
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<td>0.0001</td>
</tr>
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<td>uPA</td>
<td>uPAR</td>
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<td>0.0001</td>
</tr>
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<td>PAI-1</td>
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</tr>
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<td>IL-8</td>
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</tr>
<tr>
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<td>TNF-α</td>
<td>0.24</td>
<td>0.001</td>
</tr>
<tr>
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<tr>
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<td>CAP18</td>
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<td>0.01</td>
</tr>
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<tr>
<td>IL-10</td>
<td>IFN-γ</td>
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</tr>
<tr>
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<td>CAP18</td>
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<td>0.0001</td>
</tr>
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<td>FEV₁</td>
<td>PAI-1</td>
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<tr>
<td>FEV₁</td>
<td>uPAR</td>
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</tbody>
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**Table 3—Schematic Representation of Disease-Specific Patterns of Innate Immune Factors in Sputum as Compared to Healthy Control Subjects**

<table>
<thead>
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<th>Factors</th>
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<th>Asthma</th>
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<tr>
<td>uPA</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>uPAR</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>PAI-1</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

* ↑ = elevated; ↓ = depressed; ↔ = equivalent.
structure; positive ions, pH, and its own concentration affect the structure and change its antimicrobial activity.\textsuperscript{54} It is unknown how changes in pH, ion concentration, sputum viscosity, and other factors in patients with chronic airways disease may affect the bioactivity of CAP18. We found that increases in CAP18 correlated with IL-10 and IFN-\gamma rather than IL-8 or TNF-\alpha levels in CF, suggesting a possibly compensatory antiinflammatory rather than proinflammatory role. However, high concentrations of CAP18 may be cytotoxic to eukaryotic cells, and a deleterious effect certainly cannot be excluded.\textsuperscript{54}

Sputum levels of PAI-1 also correlated negatively with pulmonary function. This suggests a relationship between PAI-1 and the degree of inflammation and tissue remodeling. PAI-1 binds uPA/uPAR, forming uPA/uPAR/PAI-1 complexes that are internalized across the cell membrane together with low-density lipoprotein receptor-related protein and degraded in the lysosome. uPAR is recycled to the cell membrane. PAI-1 thus not only controls the proteolytic activity of uPA but also modulates the number of uPAR on the cell surface.\textsuperscript{15,55} The parallel increase of PAI-1 with uPAR suggests a finely tuned proteolytic balance is critical in the airway plasminogen activator system.\textsuperscript{50} The dissolution and remodeling of extracellular matrix depends on a tightly controlled dynamic that maintains a proper balance between uPA/uPAR and PAI-1.\textsuperscript{57} The increase in the PAI-1 may be homeostatic for the proproteolytic activity of increased uPAR.

In conclusion, comparison of CF to COPD and asthma as well as normal control subjects revealed interesting differences in innate immune factor levels. CAP18 is elevated in mild CF as well as COPD and inversely related to lung function, but correlation to IL-10 and IFN-\gamma suggests it may homeostatic rather than proinflammatory. The low levels of CAP18 in asthma are unexpected, perhaps reflecting the eosinophilic character of asthmatic inflammation or metabolic differences. Elevations of IL-8 in CF sputum, and IFN-\gamma in sputum CAP18 (elevated in CF and COPD, depressed in asthma) suggest further studies on the role of cathelicidins in specific forms of airway inflammation. The practical implications of such studies will likely emerge only after considerably more investigation.

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