Novel Neutrophil-Derived Proteins in Bronchoalveolar Lavage Fluid Indicate an Exaggerated Inflammatory Response in Pediatric Cystic Fibrosis Patients

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Background: Airway inflammation in cystic fibrosis (CF) is exaggerated and characterized by neutrophil-mediated tissue destruction, but its genesis and mechanisms remain poorly understood. To further define the pulmonary inflammatory response, we conducted a proteome-based screen of bronchoalveolar lavage fluid (BALF) collected from young children with and without CF experiencing endobronchial infection.

Methods: We collected BALF samples from 45 children younger than 5 years and grouped them according to the presence of respiratory pathogens: >1 × 105 colony-forming units (CFU)/mL BALF (18 and 12 samples with and without CF, respectively) and <1 × 105 CFU/mL (23 and 15 samples). BALF proteins were analyzed with SELDI-TOF mass spectrometry (MS) and H4 Protein-Chips®. Proteins were identified and characterized using trypsin digestion, tandem MS, Fourier transform ion cyclotron resonance MS, immunoblotting, and ELISA.

Results: The SELDI-TOF MS BALF profiles contained 53 unique, reliably detected proteins. Peak intensities of 24 proteins differed significantly between the CF and non-CF samples. They included the neutrophil proteins, α-defensin 1 and 2, S100A8, S100A9, and S100A12, as well as novel forms of S100A8 and S100A12 with equivalent C-terminal deletions. Peak intensities of these neutrophil proteins and immunoreactive concentrations of selected examples were significantly higher in CF than non-CF samples.

Conclusions: Small neutrophil-derived BALF proteins, including novel C-terminal truncated forms of S100A proteins, are easily detected with SELDI-TOF MS. Concentrations of these molecules are abnormally high in early CF lung disease. The data provide new insights into CF lung disease and identify novel proteins strongly associated with CF airway inflammation.

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Lung disease in cystic fibrosis (CF)13 is characterized by recurrent, and then persistent, bacterial infection accom-
panied by neutrophil-dominated airway inflammation that begins in the 1st years of life. Early infectious episodes usually involve *Staphylococcus aureus*, *Hemophilus influenzae*, and nonmucoid *Pseudomonas aeruginosa*, whereas mucoid strains of *P. aeruginosa* dominate by early adolescence, resulting in chronic, multiantibiotic-resistant infections and a major decline in pulmonary function (1). CF endobronchial infection is accompanied by an excessive inflammatory response, which appears to be directly related to the lack of the affected gene product, the CF transmembrane conductance regulator protein (CFTR). Although the lungs of CF patients are structurally normal at birth, signs of inflammation are apparent in bronchoalveolar lavage (BAL) fluid (BALF) of asymptomatic infants (2), and the proinflammatory cytokines interleukin (IL)-1β, IL-6, and IL-8 are found in unusually high concentrations in the airways of infected CF patients (3). Decreased concentrations of antiinflammatory molecules such as IL-10 (4) and lipoxin (5) have also been noted in older, but not in younger, CF patients and may be related instead to secondary events (6, 7). CF-associated effects on neutrophil function, including increased priming and increased production of tissue-damaging molecules and IL-8, may further help to perpetuate the problem (8, 9). Taken together, these findings suggest that mechanisms underlying the control of inflammation, as well as responses to infection, may be compromised in CF. Current clinical approaches to reducing pulmonary inflammation in CF are limited in their scope and by concerns over toxicities associated oropharyngeal culture for *P. aeruginosa*. Apart from BALF samples from young CF patients and pulmonary disease controls for disease-associated proteins, with the aim of identifying novel molecules involved in disease that may help in further understanding CF lung inflammation and potentially serve as new antiinflammatory targets.

**Materials and Methods**

**STUDY PARTICIPANTS**

We obtained 41 BALF specimens from 18 children with CF (diagnosed by gene mutation analysis and sweat chloride concentration ≥60 mmol/L) and 1 sample each from 27 children without CF (Table 1). All patients were younger than 5 years. The CF patients were participating in an ongoing multicenter, randomized controlled trial of BAL-directed therapy (Australian Clinical Trial Registry No. 1260500065639) (13). Children with CF underwent routine BAL in the 1st 6 months of life. Subsequently, BAL was also performed when they developed a persistent cough that failed to improve with standard nonantipseudomonal oral antibiotics or when there was a positive oropharyngeal culture for *P. aeruginosa*. Apart from their persistent cough, none of these children had additional lower respiratory symptoms or signs when under-

### Table 1. BALF specimen group characteristics.

<table>
<thead>
<tr>
<th>Specimen group</th>
<th>CF</th>
<th>Non-CF</th>
<th>CF</th>
<th>Non-CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient group</td>
<td>CF</td>
<td>18/11</td>
<td>CF</td>
<td>23/13</td>
</tr>
<tr>
<td>Pathogen loada</td>
<td>≥1 × 10^5 CFU/mL</td>
<td>&lt;1 × 10^5 CFU/mL</td>
<td>≥1 × 10^5 CFU/mL</td>
<td>12/12</td>
</tr>
<tr>
<td>Number of samples/patientsb</td>
<td>9/9</td>
<td>12/11</td>
<td>6/6</td>
<td>7/8</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>31.2 (20.4, 38.4)</td>
<td>20.4 (6.0, 37.2)</td>
<td>10.2 (3.6, 27.6)</td>
<td>24.0 (6.0, 50.4)</td>
</tr>
<tr>
<td>Age, monthsc</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Pathogens detected (no. of samples)d</td>
<td>P. aeruginosa (8)</td>
<td>P. aeruginosa (12)</td>
<td>Streptococcus pneumoniae (7)</td>
<td>P. aeruginosa (1)</td>
</tr>
<tr>
<td></td>
<td>S. maltophilia (3)</td>
<td>H. influenzae (7)</td>
<td>S. pneumoniae (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. aureus (6)</td>
<td>Adenovirus (1)e</td>
<td>H. influenzae (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aspergillus spp. (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moraxella catarhalis (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a BALF samples were categorized into 1 of 4 groups according to CF status, and either the bacterial/fungal load of respiratory pathogens [more or less than 10^5 CFU/mL, a previously defined threshold for infection (15)] or the presence of respiratory viruses.

b Some CF patients provided more than 1 BAL specimen to different categories.

c Median (25th and 75th quantiles).

d Samples containing respiratory pathogens ≥1 × 10^5 CFU/mL (groups 1 and 3) or between 1 × 10^2 and <1 × 10^5 CFU/mL (groups 2 and 4).

e Virus detected, but not quantified.
going BAL. Control patients (n = 27) had a chronic cough and/or stridor for at least 6 weeks and were undergoing routine flexible bronchoscopy with BALF analysis as part of a series of investigations to determine the underlying diagnosis. In 17 patients, airway lesions were present, including tracheomalacia (n = 9), bronchomalacia (n = 6), or laryngomalacia (n = 8); 2 patients had bronchiectasis, 4 had evidence of bronchitis with focal edema and purulent secretions, and 4 received a final diagnosis of chronic nonspecific cough of childhood (14). All the BALs were performed (with informed consent) at the Royal Children’s Hospitals in Brisbane (all CF samples and 25 of 27 control samples) and Melbourne (2 of 27 control samples) between 1999 and 2005.

The samples were categorized into 1 of 4 groups according to CF status and the bacterial/fungal load of respiratory pathogens in the BALF associated with their symptoms of endobronchitis (Table 1) (15). Some samples had multiple pathogens present. No significant differences in bacterial load were noted between specimen groups 1 and 3 or 2 and 4. Because of the longitudinal nature of the trial, some CF patients underwent repeated bronchoscopy and so contributed multiple samples to each group. Specifically, 11 children contributed samples to the $\geq 1 \times 10^5$ colony-forming units (CFU)/mL group (1 child provided 6 samples, 1 provided 3, and 9 provided 1 each), and 13 contributed samples to the $< 1 \times 10^5$ CFU/mL group (2 provided 3, 6 provided 2, and 5 provided 1 each). Patient age and sex distributions did not differ substantially between any of the groups (Table 1).

**BALF COLLECTION**

BALF collection was standardized across both centers according to our previously described technique (16), with minor modifications. Briefly, with the patient under sevoflurane anesthesia, the vocal cords were sprayed with 1% lidocaine and then a flexible bronchoscope (via laryngeal mask) was introduced sequentially into the right middle lobe and lingula bronchi. Nonbacteriostatic normal saline was instilled through the bronchoscope suction channel (1 mL/kg; maximum 20 mL) into the distal bronchus and immediately aspirated into a sterile 40-mL Argyle specimen trap (Tyco Healthcare Group LP). The lavage was repeated, and the BALF sample was pooled, placed in an insulated transport container with $-20 \degree$C ice packs, and immediately sent to the laboratory for processing. All samples were processed in a standard manner within 60 min. This involved the removal of an aliquot for cytology and microbiology analysis, and centrifugation of the remainder at 500g for 5 min. Supernatants were then transferred into polypropylene tubes (Nalge Nunc International) and immediately placed at $-80 \degree$C for storage.

**BALF ANALYSIS**

*Microbiology and cytology.* We performed total cell and differential cell counts and identified bacterial, fungal, and mycobacterial pathogens from 500 $\mu$L of BALF by standard methods reported elsewhere (16, 17). We conducted direct immunofluorescence antigen testing for respiratory viruses on BALF as described (16); if results were negative, we performed nucleic acid amplification tests using a multiplex reverse transcriptase-PCR assay (18). Final counts for bacterial and fungal pathogens were expressed as CFU per mL of BALF. The lower limit of detection was 100 CFU/mL. Except for an adenovirus detected in 1 disease control sample, no common viral pathogens or mycobacterial species were detected.

*Biochemical assays.* BALF was analyzed using ELISA for concentrations of IL-8 (BioSource) and $\alpha$-defensin (DEFA; HyClone Biotechnology), and lysozyme activity was measured using an EnzCheck Lysozyme Activity Kit (Molecular Probes), all according to manufacturers’ instructions. Limits of detection were 0.7 ng/L, 50 ng/L, and 20 kU/L, respectively. We measured BALF protein concentrations by use of a bicinechonic acid assay reagent set (Pierce) and BSA calibrators.

**SELDI-TOF MS ANALYSIS**

Frozen BALF specimens were thawed, mixed with 0.5× Complete Protease Inhibitor (Roche Diagnostics), divided into aliquots, and refrozen at $-80 \degree$C. Two aliquots per sample were analyzed (on separate days) using H4-ProteinChips® (C18-coated surface) and a PBS II instrument (both from Ciphergen Biosystems). Spectra were collected at 3 different optimized $m/z$ ranges (2000–15 000, 8000–30 000, and 20 000–100 000) and normalized by total ion current, excluding matrix-associated signal ($m/z < 2000$). Spectra were aligned, and peaks (clustering within a 0.3% $m/z$ window) with intensities $> 3$ in at least 5% of all BALF sample spectra were quantified using BiomarkerWizard® software (Ciphergen Biosystems). Other details can be found in the Methods in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue10.

**FOURIER TRANSFORM ION CYCLOTRON RESONANCE MS ANALYSIS**

We performed Fourier transform ion cyclotron resonance (FTICR)-MS by use of an APEX-Qe Qq-FTICR instrument (Bruker Daltonics) equipped with a 12-Tesla actively shielded magnet and an Apollo II (Bruker Daltonics) electrospray ionization source. Lyophilized HPLC fractions containing BALF proteins were resuspended in 100 $\mu$L deionized water or water containing 0.1% formic acid, and the fractions were further diluted with electrospray solvent (50% acetonitrile, 0.1% formic acid, or 10 mmol/L ammonium acetate). Analyses were carried out by direct infusion using a syringe pump (flow rate of 200 $\mu$L/min). For top-down analyses, multiply charged ions of the intact proteins were selected in the quadrupole for collision-induced dissociation, which was performed in the collision cell in the presence of argon as the collision gas.
The data were analyzed using Biotools data software from Bruker Daltonics and manual interpretation.

**IMMUNOBOTTING**
BALF samples (15 μg total protein) were separated using denaturing SDS-PAGE and tricine/20% acrylamide gels and electroblotted to nitrocellulose membrane (Schleicher & Schuell). Anti-S100A8 antibody (from C. Sorg, University of Munster, Munster, Germany) and secondary antibody, antirabbit horseradish peroxidase conjugate, were diluted (1/2000 and 1/10 000, respectively) in PBS (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na2HPO4, and 0.0018 M KH2PO4) containing, per liter, 50 g skim milk powder and 1 mL Tween-20. Chemiluminesence (SuperSignal Chemiluminescent Substrate, Pierce), autoradiography, and densitometry (GS-800 Densitometer, Bio-Rad) were used to determine relative quantities of the immunoreactive bands matching the migration of S100A8 monomers.

**STATISTICAL METHODS**
We compared cytology results and IL-8 concentrations between prespecified pairs of sample groups, namely 1 vs 2, 3 vs 4, 1 vs 3, and 2 vs 4 (see Table 1), using a modified t-test, a regression method with robust information-sandwich standard errors to allow for repeated measures due to the same infants contributing samples to more than one group. These analyses were performed in the log scale for concentration values, because of the positively skewed distributions. We used the same modified t-test approach to compare SELDI-TOF MS spectral peak intensities, also in the log scale, between the patient groups. We adjusted for multiple comparisons using a false-discovery rate threshold of q < 0.05 (19). Group 1 (Table 1) samples with (n = 8) and without (n = 10) *P. aeruginosa* were also compared for cytology results, IL-8 concentrations, and SELDI-TOF MS spectral peak intensities.

We used Spearman rank correlation to assess the association between selected spectral peak intensities and neutrophil concentrations. Statistical analysis was performed using the Stata package (release 9.2; Stata Corporation).

**RESULTS**
**IL-8 AND INFLAMMATORY CELL PROFILES**
We compared the types and numbers of cells and concentrations of IL-8 in the BALF samples between the 4 specimen groups (see Table 1) to examine associations with CFTR function (group 1 vs 3 and 2 vs 4) and pathogen load (group 1 vs 2 and 3 vs 4). Fig. 1 illustrates the most abundant cell types for these groups (neutrophils, macrophages, and epithelial cells), together with IL-8 concentrations (see Fig. 1 in the online Data Supplement for other cells). Non-CF samples with ≥1 × 10^5 CFU/mL pathogens had significantly more neutrophils [geometric mean (GM) ratio 2.9, 95% CI 0.6–5.3, P = 0.017] and higher IL-8 concentrations (GM ratio 3.1, 95% CI 1.3–5.0, P = 0.002) than non-CF samples with <1 × 10^5 CFU/mL pathogens. Also, an adjusted comparison of IL-8 concentrations between these groups showed that IL-8 differences were not explained by adjusting for differences in neutrophils (although the adjusted association was reduced). Significantly higher epithelial cells numbers (GM ratio 1.8, 95% CI 0.9–2.7, P < 0.001) and IL-8 concentrations (GM ratio 3.1, 95% CI 1.6–4.6, P < 0.001) were found in CF samples containing <1 × 10^5 CFU/mL pathogens compared with group 4 non-CF samples. Additionally, within group 1 there was no evidence of differences in cytology and IL-8 concentrations between specimens with and without *P. aeruginosa*, although these comparisons had limited power because of the small numbers.

**SELDI-TOF MS ANALYSIS OF BALF AND IDENTIFICATION OF DISEASE-ASSOCIATED PROTEINS**
SELDI-TOF MS spectra generated from the BALF samples typically displayed proteins ranging between 2500 and 20 000 Da. Two particularly abundant clusters of proteins were located at approximately 3.4 kDa and 10–13 kDa (Fig. 2). Comparing across all the samples, 102 peaks representing at least 53 unique proteins (taking into account redundancy due to overlapping spectrum ranges and multiple-charged peaks) were reliably detected. Statistical comparisons of all peak intensities between the respective CF and non-CF groups (group 1 vs 3 and 2 vs 4) indicated that 24 of 53 proteins were significantly different (P < 0.05; see Table 1 in the online Data Supplement). Fifteen were expressed more highly in one or both of the CF groups and 9 more highly in the control groups. Notable examples included a 10 580-Da species with at
least 6-fold higher mean peak intensity in the CF group 1 vs non-CF group 3 samples (\( P < 0.001 \)) and a 3086-Da species that was almost 7-fold lower in the CF group (\( P < 0.001 \)). Comparison between bacterial loads within each patient type indicated that 4 of these proteins were significantly higher in group 1 than group 2 CF samples, but no differences were observed between the non-CF samples. Additionally, within group 1 no differences in

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**Fig. 2. BALF SELDI-TOF MS spectra.**

(A), representative example of a SELDI-TOF MS spectrum from a group 1 BALF sample. Two mass regions are depicted, illustrating the positions and relative intensities of peaks subjected to further analysis. (B), gel view representations of SELDI-TOF MS spectra for all the study samples. The same mass regions as in (A) are shown, and samples are grouped into the 4 specimen groups. Spectra for each group are ranked according to total neutrophil number (highest to lowest).
peak intensities were detected between specimens with and without *P. aeruginosa* (with a false-discovery rate threshold of *q* < 0.05).

Using a combination of strategies involving fractionation (reversed-phase HPLC and/or 1-dimensional SDS-PAGE), MS-MS analysis of trypsin-treated fractions, N-terminal sequencing, and/or FTICR-MS analysis, we identified 11 of the proteins associated with significant changes in expression. Table 2 summarizes the protein identities and approaches used for each assignment. The 3445- and 3373-Da peaks (Fig. 2A, peaks 2 and 1, respectively) were identified as neutrophil-derived proteins DEFA1 and 2. An additional DEFA with a molecular mass of 3485 Da was identified as DEFA3 (Fig. 2A, asterisk). It was absent in a subset of patients (4 of 18 CF and 5 of 27 non-CF), consistent with a genetic polymorphism that was absent in a subset of patients (4 of 18 CF and 5 of 27 non-CF). The 2 DEFA with a molecular mass of 3485 Da was identified as DEFA3 (Fig. 2A, asterisk). It was absent in a subset of patients (4 of 18 CF and 5 of 27 non-CF).

### Table 2. Identification evidence for BALF proteins detected by SELDI-TOF MS.

<table>
<thead>
<tr>
<th>Spectrum peak number</th>
<th>Protein identity</th>
<th>Swissprot number</th>
<th>Observed mass</th>
<th>Actual mass</th>
<th>Tryptic peptide</th>
<th>MS-MS score</th>
<th>N-terminal sequence</th>
<th>FTICR-MS (C-terminus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEFA2</td>
<td>P59665</td>
<td>3373</td>
<td>3375.5</td>
<td>6</td>
<td>6 (75%)</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>DEFA1</td>
<td>P59665</td>
<td>3445</td>
<td>3346.6</td>
<td></td>
<td>6 (73%)</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>S100A12*</td>
<td>P80511</td>
<td>10220</td>
<td>10186.5</td>
<td>3 (37%)</td>
<td>TKLEEH</td>
<td>. . .</td>
<td>HYTHKE</td>
</tr>
<tr>
<td>4</td>
<td>S100A12</td>
<td>P80511</td>
<td>10445</td>
<td>10444.9</td>
<td>8 (47%)</td>
<td>MLTELE</td>
<td>. . .</td>
<td>KSHEESH</td>
</tr>
<tr>
<td>5</td>
<td>S100A9*</td>
<td>P05109</td>
<td>10580</td>
<td>10578.3</td>
<td>8 (50%)</td>
<td>MLTELE</td>
<td>. . .</td>
<td>HEESH</td>
</tr>
<tr>
<td>6</td>
<td>S100A9</td>
<td>P05109</td>
<td>10832</td>
<td>10835.6</td>
<td>9 (72%)</td>
<td></td>
<td></td>
<td>232</td>
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<tr>
<td>7</td>
<td>S100A9*</td>
<td>P06702</td>
<td>12689</td>
<td>12690.3</td>
<td>9 (53%)</td>
<td></td>
<td></td>
<td>126</td>
</tr>
<tr>
<td>8</td>
<td>DEFA1</td>
<td>P59665</td>
<td>3445</td>
<td>3346.6</td>
<td>3 (20%)</td>
<td></td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>9</td>
<td>DEFA2</td>
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<td>3375.5</td>
<td></td>
<td></td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>10</td>
<td>DEFA3</td>
<td>P61626</td>
<td>14686</td>
<td>14701.8</td>
<td>5 (35%)</td>
<td></td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>11</td>
<td>Lysozyme</td>
<td>P68871</td>
<td>15871</td>
<td>15868.3</td>
<td>3 (20%)</td>
<td></td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>12</td>
<td>β-Globin</td>
<td>P68871</td>
<td>15871</td>
<td>15868.3</td>
<td></td>
<td></td>
<td></td>
<td>55</td>
</tr>
</tbody>
</table>

*a* Peak numbers as defined in Fig. 2A.

*b* Average observed mass determined by SELDI-TOF MS.

*c* Predicted monoisotopic mass (Da).

*d* Number of tryptic peptides identified by MS-MS that match those predicted (percentage of coverage of the protein sequence).

*e* MOWSE score (generated using the MASCOT search tool) of the tryptic peptides subjected to MS-MS fragmentation analysis.

*f* Peptide sequence determined by N-terminal sequencing of proteins purified by SDS-PAGE.

*g* C-terminal peptide sequence determined by FTICR-MS analysis.

14 Human gene: *DEFA3*, defensin, alpha 3, neutrophil-specific.
DEFA1–3 concentrations, quantified by ELISA, ranged between 5 and 2000 μg/L, similar to concentrations found in other pulmonary disease patient samples (22). Respective cumulative peak intensities correlated with sample concentrations ($r = 0.62$, $P = 0.00006$; Fig. 4F). Likewise, lysozyme peak intensities correlated strongly with enzyme activity levels measured in the BALF samples ($r = 0.81$, $P < 0.0001$).

Some of the neutrophil-derived proteins present in the SELDI-TOF MS profiles were also associated with neutrophil numbers in each patient group. Namely, DEFA1 [Spearman correlation for CF and non-CF ($P$ value), 0.28 (0.10) and 0.56 ($<0.01$)], DEFA2 [0.38 (0.02) and 0.50 (0.01)], S100A8 [0.33 (0.05) and 0.59 ($<0.01$)], S100A8* [0.26 (0.12) and 0.51 (0.01)], and S100A12 [0.29 (0.08), 0.49 (0.01)].

**Discussion**

Much evidence suggests that the lower airway inflammation seen in CF is excessive and dependent on CFTR function, although its genesis remains poorly understood. We were able to investigate the early stages of this phenomenon at the proteomic level through the use of a relatively sensitive and high-throughput analytical method, SELDI-TOF MS, and BALF samples from children with and without CF. The children were of similar age and care center location and underwent the same BALF collection methods. In addition, proteomic analysis comparing samples with and without *P. aeruginosa* did not detect any differences according to bacterial species. Therefore, although CF samples were more likely to be infected with *P. aeruginosa*, the presence of this organism is unlikely to have contributed to differences between the groups. The recruitment into the study population of young children with CF before the onset of chronic *P. aeruginosa* infection was particularly important for elucidating disease genesis because we were able to address several potential confounding variables and demonstrate a significant contribution of CFTR function to the BALF proteome.

The use of SELDI-TOF MS was a particularly powerful tool with which to address these questions. Only minute amounts of sample were required to derive, simultaneously and relatively accurately, the concentrations of many different proteins. Significant numbers of samples could also be screened rapidly and reproducibly, allowing consistent changes to be distinguished from sample-to-sample variation. We were able to partly circumvent the major drawback of SELDI-TOF MS, subsequent protein identification, through the use of FTICR-MS, which is ideally suited to the identification of typically small (<20 kDa) proteins detected by SELDI-TOF. Unfortunately, the limited availability of non-CF samples precluded the identification of the proteins most strongly associated with this group. Although others have shown that approximately 1500 different proteins may be present in BALF (12), the relatively low number we observed by use
of SELDI-TOF MS may actually represent significant coverage given the method’s restricted detection range. The most readily detected and identifiable proteins in the BALF were DEFA and S100A proteins. Given their likely source, activated neutrophils, their functions (discussed below), and that concentrations were correlated with neutrophil numbers, these proteins may become useful as surrogate markers of inflammation. The properties particular to these molecules, namely their small size, high stability, and low pI, make them especially amenable to SELDI-TOF MS analyses. Indeed, these proteins have been detected by this approach in a range of other clinical samples in which inflammation and/or infection were present (23, 24).

Interestingly, we observed significantly higher concentrations of these proteins in CF than non-CF BALF samples. This observation has important implications for understanding CF endobronchial inflammation, because these proteins are known to modulate several aspects of this process. DEFA proteins are chemotactic for monocytes (25), stimulate the production of proinflammatory cytokines from monocytes and endothelium (26), and act on the recruitment, proliferation, and maturation of T cells (27). Increased DEFAs may also contribute to tissue damage directly via their observed cytotoxic effects on cells (28), and indirectly by competing with neutrophil elastase (NE) for binding of the NE inhibitor, α1-antitrypsin (29). Indeed, increased free NE activity is associated with CF inflammation and lung damage (30, 31). S100A8/A9 heterodimer is chemotactic for neutrophils in vitro and in vivo (32). S100A12 induces the expression of endothelial adhesion molecules, promoting leukocyte recruitment (33), neutrophil oxidative burst, degranulation and secretion (34), and pulmonary mast cell activation (35). Therefore, higher than normal concentrations of the S100A and DEFA molecules in the CF lung likely contribute to the excessive proinflammatory environment through multiple processes, potentially inhibiting resolution of inflammation and allowing tissue damage to occur. With respect to why these proteins are more...
abundant in CF, other studies have shown that CF patient–derived neutrophils (both circulating and peripheral) were more sensitive to cytokine stimuli (8), had a greater oxidant-producing capacity (36), and secreted more proinflammatory molecules than cells from controls (9). Others have also observed production of these proteins from epithelial cells (22, 37, 38), the major site of CFTR function.

We discovered novel truncated forms of the S100A8 and A12 proteins that were more strongly associated with CF than disease control samples. It remains to be determined how they are formed, but the absence of additional immunoreactive bands in the immunoblot analysis indicates that nonspecific protein degradation is unlikely. The cleavage site and cleaved amino acids involved (H-KE) are identical in each protein, raising the question of a yet-to-be defined proteolytic activity upregulated in the CF lung. Excessive concentrations of proteases have been described in the CF lung (30, 31), and lung-associated proteases can specifically target murine S100A proteins (39). The C-terminal truncations may also have consequences on protein function. Residues located near the C-terminus of S100A12 are predicted to mediate S100A12–receptor recognition (40).

In conclusion, this study demonstrates the utility and power of proteome profiling technologies in the analysis and understanding of CF lung disease. Our use of well-defined groups of BALF samples, in conjunction with the ability to generate robust proteomic profiles of several proinflammatory related molecules, provide additional evidence that inflammatory processes are exaggerated in CF. The data also provide additional molecular detail regarding the CF inflammatory state, including the presence of novel S100 proteins that are strongly associated with CF pulmonary infection.

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References


