ORIGINAL RESEARCH

André M. Cantin, MD²
Ginette Bilodeau, BSc²
Pierre Larivée, MD²
Martin V. Richter, PhD²

Respiratory Division, Department of
Medicine, Centre Hospitalier Universitaire
de Sherbrooke.

Plasma biomarkers and cystic fibrosis lung disease

Abstract

Purpose: The purpose of this study was to determine whether plasma biomarkers reflect changes in lung function and respiratory exacerbations associated with CF lung disease.

Methods: Plasma human leukocyte elastase/alpha1 antitrypsin complex (pHLE complex) values were measured in 28 adult CF patients and 47 healthy volunteers and correlated with forced expiratory volume (FEV1) and forced vital capacity (FVC). pHLE complexes were studied during respiratory exacerbations and after antibiotic therapy. Plasma cytokines and sialic acid were also measured.

Results: pHLE complexes were increased in CF patients ($p < 0.01$), were inversely correlated with FEV1 ($r = 0.71$) and FVC ($r = 0.67$) and returned to normal levels after intravenous antibiotics ($p < 0.001$). Plasma cytokines did not correlate with lung function. Total sialic acid increased during CF respiratory exacerbations and decreased after antibiotic therapy.

Conclusion: Plasma sialic acid and pHLE complexes reflect clinically meaningful changes in CF lung disease. In contrast, plasma cytokine levels did not correlate with lung function.

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Correspondence to:

André M. Cantin, M.D.
Respiratory Division, Department of Medicine
Centre Hospitalier Universitaire de Sherbrooke
3001 12ième Avenue Nord, Sherbrooke, Quebec, J1H 5N4
Phone: (819) 346-1110 ext. 14881
E. mail: Andre.Cantin@USherbrooke.ca

Most patients with cystic fibrosis (CF) develop severe bronchiectasis, which ultimately leads to respiratory failure. CF is a potentially fatal disease caused by a single gene defect [1-3]. This defective gene encodes the cystic fibrosis trans-membrane conductance regulator protein or CFTR [4-6]. New therapies addressing this defect are being tested in clinical trials [7, 8]; however, quantifying clinical benefits remains a major challenge. Plasma biomarkers may represent useful tools for monitoring CF lung disease and patient response to therapy.

Bacteria in the lower respiratory tract of patients with CF induce a systemic inflammatory response and a sustained recruitment of neutrophils from peripheral blood [9-14]. The CF lung inflammatory response is characterized by the release of several cytokines within the airways and a systemic increase in acute phase reactants [15, 16]. Recently developed cytokine assays, based on ELISA or cytokine bead arrays, allow the measurement of multiple cytokines in small sample volumes, offering the possibility of exploring new blood biomarkers in patients with CF. Furthermore, total sialic acid has been reported to be a robust inflammatory marker that tracks the acute phase response in several diseases [17]. Acute phase reactants and airway mucins are heavily sialated glycoproteins that increase in patients with CF. We therefore hypothesized that total sialic acid in plasma may be increased during acute and chronic lung inflammation associated with CF. The potential of plasma cytokine array assays and total sialic acid to assess CF lung disease was evaluated.

Neutrophils bring with them potentially toxic products that are released into the airways. Among these products, human leukocyte elastase (HLE: EC3.4.21.37) has a particularly broad range of deleterious effects directly relevant to the pathogenesis of CF lung disease [18]. HLE overwhelms its natural inhibitor, α 1 antitrypsin (α 1AT), in the airway of CF patients. Active HLE, from the respiratory epithelial surface, spills over into the blood where it is rapidly inactivated by α 1AT and forms a complex that can be detected in the plasma. HLE and α 1AT complexes (pHLE complexes) are increased in CF plasma, suggesting that neutrophil granule products may represent useful markers of CF pulmonary inflammation and infections [19-22]. Despite these and other reports, the pHLE complex has not been widely used as a biomarker for CF lung disease.

The aim of this study was to assess plasma cytokines, total sialic acid and pHLE complexes as possible biomarkers of CF lung disease during periods of stable respiratory health without exacerbation and in patients treated for acute respiratory exacerbations with intravenous antibiotic therapy.

Methods

Study subjects

Twenty-eight patients with CF (age: 25.9 ± 6.2 years, 3 smokers) and 47 healthy individuals (age: 33.6 ± 11.8 years, 9 smokers) were recruited. Of the 28 recruited subjects with CF, 16 were studied only during a stable period, six during periods of both stability and respiratory exacerbation, and six only during an acute respiratory exacerbation defined according to criteria established at the 1994 Cystic Fibrosis Foundation Microbiology and Infectious Disease Consensus Conference and published by Aaron et al. [23]. Ten patients with exacerbation were also studied on the 14th day of intravenous antibiotic therapy with two agents active against *Pseudomonas aeruginosa*. Bacteria were cultured from the sputum of all patients, and identified as follows: 21 *Staphylococcus aureus*, 19 *Pseudomonas aeruginosa*, five *Hemophilus influenzae*. This study was approved by the ethics review board of the Centre Hospitalier Universitaire de Sherbrooke. Informed consent was obtained from all subjects prior to recruitment to the study.

Plasma inflammatory cytokine assay

The Multi-analyte ELISArray kit (SABiosciences Corporation, Frederick, MD) was used according to the manufacturer's instructions to measure the following cytokines in plasma: IL1 α , IL1 β , IL2, IL4, IL6, IL8, IL10, IL12, IL17 α , IFN γ , TNF α and GM-CSF.

Plasma sialic acid assay

Plasma total sialic acid was determined using a colorimetric assay [24]. Two hundred μ l of freshly obtained plasma were added to 1.5 ml 5% perchloric acid and heated to 100° C for 5 min to release protein-bound sialic acid. Samples were cooled to RT, and centrifuged at 2,500 x g for 4 min. A 100 μ l volume of Ehrlich's reagent [25] was added to 500 μ l clear supernatant, heated to 100° C for 15 min, cooled and diluted with 500 μ l H₂O. Sample absorbance was measured at 525 nm. Concentrations were determined from a standard curve of sialic acid (N-acetylneuraminic acid, Calbiochem, San Diego, CA).

Blood sample handling and pHLE complex ELISA assay

Nine ml of blood was immediately added to 1 ml of Special Anticoagulant Solution (American Diagnostica, Stamford, CT) containing trisodium citrate, heparin, hirudin, aprotinin and sodium azide. The samples were centrifuged at 500 x g for 20 min at RT. The supernatants were collected and frozen at -20 °C. Human polymorphonuclear elastase was assayed using

a commercial ELISA kit (BMS269, Bender MedSystems GmbH, Vienna, Austria), which detects the HLE-AT complex regardless of whether the complexes form at the site of HLE release or after free HLE enters the plasma.

Lung function testing

Spirometry was performed according to the American Thoracic Society/European Respiratory Society guidelines [26] using a Jaeger Masterscope pneumotachograph (Viasys Healthcare GmbH, Höchberg, Germany). All spirometry measures were obtained during periods when patients were without respiratory exacerbation.

Statistical analysis

A one-group analysis of variance (ANOVA) followed by Bonferroni post test for all pairs was used to analyze data from multiple groups. A paired t-test was used to analyze the effect of antibiotic therapy. Linear regression and two-tailed P values were computed based on Pearson correlation calculations. Receiver-operator characteristic (ROC) curve analysis was applied to the pHLE complex data from control and stable CF patients. All statistical analyses were performed using Prism 4 software (GraphPad Software Inc., La Jolla, CA). Data are expressed as the mean $\pm$ SD, and differences were considered statistically significant when the value of P was < 0.05 .

Results

Plasma cytokines

No significant differences in plasma cytokines (IL1 α , IL1 β , IL2, IL4, IL6, IL8, IL10, IL12, IL17 α , IFN γ , TNF α and GM-CSF) were detected among patients with stable cystic fibrosis and healthy volunteers or patients with respiratory exacerbations (data not shown). The lower limit of detection was 1 – 10 pg/ml, depending upon the cytokine measured. The number of subjects with levels below this detectable limit was similar in healthy volunteers (10.9%) and CF patients (7.4%). Although IL6 tended to be elevated in all patients with CF, the differences were not statistically significant [Figure 1A]; however, 14 days of antibiotic therapy during a respiratory exacerbation induced a statistically significant decrease in plasma IL6 in CF patients (Figure 1B, 51.3 ± 21.2 versus 20.2 ± 11.8 pg/ml, $P < 0.05$).

Plasma sialic acid

Plasma sialic acid concentrations were detectable in all healthy volunteers (Figure 2A 0.95 ± 0.21 mM). Although plasma sialic acid concentrations were not significantly elevated in patients with stable CF (1.31 ± 0.28 mM), the concentrations were elevated during respiratory exacerbations (Figure 2A, 1.78 ± 0.43 mM, $P < 0.001$ versus control). Plasma sialic acid was significantly decreased after intravenous antibiotic therapy (Figure 2B, 1.2 ± 0.2 mM, $P < 0.01$ versus exacerbation).

FIGURE 1. Plasma interleukin 6 (IL6) concentrations in healthy volunteers (control, $n = 9$), and patients with cystic fibrosis (white bars) who were stable ($n = 8$), or undergoing a respiratory exacerbation ($n = 9$). (B) Effect of 14 days of intravenous antibiotic therapy on plasma IL6 concentrations in subjects with a respiratory exacerbation of cystic fibrosis ($n = 9$). Mean $\pm$ SD, * $p < 0.05$.

FIGURE 2. Plasma total sialic acid concentrations in healthy volunteers (control, $n = 10$) and patients with cystic fibrosis (white bars) who were stable ($n = 9$) or undergoing a respiratory exacerbation ($n = 10$). (B) Effect of 14 days of intravenous antibiotic therapy on plasma sialic acid concentrations in subjects with a respiratory exacerbation of cystic fibrosis ($n = 10$). Mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGURE 3. Plasma human leukocyte elastase (pHLE) complex concentrations in healthy volunteers (control, $n = 47$) and patients with cystic fibrosis (white bars). Plasma samples were obtained when patients were stable ($n = 22$) and at the time of an acute respiratory exacerbation ($n = 12$). (B) Effect of 14 days of intravenous of antibiotic therapy on plasma pHLE complex concentrations in subjects with cystic fibrosis ($n = 10$). Mean $\pm$ SD, ** $p < 0.01$, *** $p < 0.001$.

pHLE complexes in CF

pHLE complexes were detectable in all healthy volunteers and did not vary with age or smoking history (active smokers 33.6 ± 2.9 pg/ml versus non-smokers 33.4 ± 2.0 pg/ml, $P = 0.57$). Patients with stable CF lung disease had higher levels of pHLE complexes than did control subjects (Figure 3A; 51.5 ± 17.9 pg/ml versus 33.8 ± 10.8 pg/ml, $P < 0.01$). The pHLE complex concentrations were clearly and significantly increased in patients with CF during respiratory exacerbations (111.4 ± 38.4 pg/ml, $P < 0.001$) compared with patients with stable CF and controls. All patients with CF respiratory exacerbations demonstrated a marked decrease in pHLE complexes at the 14th day of intravenous antibiotics to levels that were not significantly different from those of the healthy volunteers (Figure 3B; 44.0 ± 15.3 pg/ml, $P < 0.001$ versus exacerbation).

Receiver-Operator Characteristic (ROC) curve analysis

ROC curve analysis was used to determine the sensitivity and specificity of pHLE complex in patients with stable CF lung disease [Figure 4]. The area under the curve (AUC) for pHLE complex was 0.82 with a 95% confidence interval of 0.70 to 0.94 ($P < 0.0001$). A pHLE complex cut-off concentration of 38.9 pg/ml was associated with 77% sensitivity and 80% specificity.

FIGURE 4. Receiver-operator characteristic curve for pHLE complex. Closed circles indicate pHLE complex in stable CF patients. The solid line represents an AUC = 0.5.

FIGURE 5. Pulmonary function tests and pHLE complex concentrations in subjects with cystic fibrosis studied during stable lung disease. Correlations are shown between pHLE complex and (A) forced expiratory volume in 1 second (FEV1) and (B) forced vital capacity (FVC). FEV1, $r = 0.71$; FVC, $r = 0.67$.

pHLE complexes and lung function

An inverse correlation between pHLE complexes and lung function was present in CF patients studied during periods of stable lung disease (Figure 5A; FEV1 $r = 0.71$, $P = 0.0002$, Figure 5B; FVC $r = 0.67$, $P = 0.0006$).

Discussion

Levels of plasma IL6, total sialic acid and pHLE complexes were found to reflect responses to intravenous antibiotics in CF patients treated for respiratory exacerbations. The concentrations of all other cytokines in plasma from CF patients did not differ from those determined in healthy subjects. In contrast, sialic acid and pHLE complex were elevated in plasma from CF patients, with pHLE complex giving the most robust signal in patients with stable disease, exacerbation, and after antibiotic therapy.

Marked variations in plasma concentrations of all cytokines were observed, with no significant difference between levels found in normal and CF patients. Among all the cytokines measured, only IL6 was found to decrease after 14 days of antibiotic therapy, and this decrease was not observed in all patients. Because of these results, the possible use of plasma cytokines as biomarkers of CF lung disease was not investigated further.

Patients with CF have been shown to have increased serum levels of the high molecular weight mucin, MUC1 or KL-6, and the acute phase reactant, C reactive protein [27]. Mucin-like glycoproteins and acute phase reactant glycoproteins are heavily sialated. Sialic acid is the N-acetylated derivative of neuraminic acid. Almost all sialic acid is bound to glycoproteins or glycolipids, and is released by acid treatment. Only 0.1% of total sialic acid is not bound [17]. Increased total sialic acid concentration in blood reflects an inflammatory phenotype [28]. In contrast to the cytokines, total sialic acid concentrations in the plasma of healthy subjects showed limited scatter. Plasma total sialic acid increased during respiratory exacerbations and decreased markedly and significantly after two weeks of intravenous antibiotics. The assay of total sialic acid is rapid and inexpensive and our results suggest that further studies of this potential biomarker in CF lung disease are warranted.

This study indicates that CF patients have higher levels of pHLE complexes in their plasma during periods of stable lung disease than do healthy volunteers; however, considerable overlap in pHLE complexes exists between individuals with and without CF, since patients with less severe lung function alterations tend to have lower pHLE complexes as indicated by the

strong correlation between decreased lung function (FEV1 and FVC) and pHLE complexes. pHLE complexes are markedly increased during respiratory exacerbations of CF, and decrease to levels found in healthy volunteers after intravenous antibiotic therapy.

Several investigators have observed that pHLE complexes are increased in the blood of patients with CF [19, 29-31]. Increases in pHLE complexes have been reported in various studies of oxidant stress, antioxidant supplementation and other aspects of CF lung disease [31-34]; however, other investigators have found that HLE complexes in the blood of CF patients do not reflect respiratory exacerbations and do not correlate with lung function [27]. Most of the previous studies were conducted using various assay systems that are not readily available. The current study was done using an ELISA assay available from a commercial source, thus facilitating the applicability and reproducibility of such studies. Several factors may contribute to discrepancies between studies including differences in patient population and sample preparation. Consistent with our results, Reeves et al. have recently reported that another neutrophil-derived marker, the $\alpha 1$ -PI/C16b complex, correlates closely with lung function and response to antibiotic therapy [35].

One of the potential limitations of our study is the analysis of multiple biomarkers, which increases the potential of observing differences by chance; however, all comparisons of pHLE complexes had a value of $p < 0.01$ making this explanation less likely.

HLE reacts with $\alpha 1$ -PI with 1:1 stoichiometry to form a stable complex that can be detected in plasma [36]. Although CF patients express normal to high systemic levels of plasma $\alpha 1$ AT, they have a marked deficiency of functional $\alpha 1$ AT in their airway secretions [37, 38]. Much of this functional deficiency in $\alpha 1$ AT is related to the molar excess of HLE relative to $\alpha 1$ AT in the CF airways [13, 14, 39, 40]. While HLE forms a complex with $\alpha 1$ AT in the lung, it is likely that some free HLE also enters the blood where it forms a complex with $\alpha 1$ AT in the plasma. The assay does not distinguish between HLE- $\alpha 1$ AT complexes formed in the lung and free HLE that subsequently forms a complex with $\alpha 1$ AT in plasma. Instead, it likely reflects the total lung HLE burden.

HLE plays a key pathogenic role in the of CF lung disease. Desmosine and isodesmosine, amino acids found exclusively in cross-linked elastin and released by HLE-mediated proteolysis, are present at increased concentrations in the urine of CF patients when compared with healthy control subjects [32, 41, 42]. Patients with CF have high levels of active HLE in their sputum particularly during infectious respiratory exacerbations

[9, 13, 40, 43]. The levels of active HLE detected in the bronchoalveolar lavage fluid of CF patients correlate significantly with pulmonary function indices of the severity of lung disease such as the FEV1 and the ratio of FEV1/FVC [14, 32, 44]. Lung secretion HLE is clearly an attractive surrogate marker for CF lung disease since it directly relates to an important pathogenic pathway in CF. Sputum is one potential source of biomarkers of CF inflammatory lung disease [45]; however sputum is inhomogeneous, is often contaminated with saliva and requires significant ex vivo manipulation. More importantly, patients with CF may be incapable of producing sufficient sputum for analysis at the required times during clinical trials. While induced sputum and bronchoalveolar lavage (BAL) are alternatives, they are less practical for routine use in clinical trials. In contrast, pHLE complexes do not have the limitations of sputum and BAL, and may prove particularly helpful in evaluating the efficacy of novel therapies for CF lung disease.

The loss of CF lung function is likely due to several factors including inflammation, mucus plugging, bronchoconstriction, bacterial infection and irreversible structural damage. The correlation between the specific inflammatory marker of pHLE complexes and lung function in stable CF patients is striking, and may reflect the major pathophysiological role that HLE plays in each one of these factors and in CF lung disease [18].

Several new CF therapeutic strategies, aimed at correcting either the basic defect of CFTR or its consequences, have been developed and need to be tested in clinical trials. Two endpoints recognized by CF clinicians and regulatory agencies to be of clinical significance in CF are lung function tests (FEV1 and FVC) and the frequency of respiratory exacerbations. Each of these endpoints has limitations. Lung function tests are prone to great variability in the course of CF lung disease. Respiratory exacerbation is a subjective measure difficult to quantify. In contrast, the total sialic acid and pHLE complex assays are simple quantitative blood tests that reflect relevant components of the pathogenic process in CF lung disease and correlate with changes that are generally recognized as clinically significant.

In summary, plasma total sialic acid and pHLE complex concentrations are demonstrated to be elevated in CF patients, particularly during respiratory exacerbations and pHLE complex correlates with lung function in CF patients during periods of stable lung disease. These biomarkers reflect both CF lung disease severity and acute exacerbations in CF and may be of particular interest in future clinical trials of novel therapies for CF lung disease.

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