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Published in:
Pediatric Pulmonology

Document Version:
Publisher's PDF, also known as Version of record

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Download date:30. Oct. 2018
The Effect of Treatment of Cystic Fibrosis Pulmonary Exacerbations on Airways and Systemic Inflammation

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Summary. Background: Chronic infection in cystic fibrosis (CF) and airway inflammation lead to progressive lung injury. Neutrophils are considered to be responsible for the onset and promotion of the inflammatory response within the CF lung. The relationship between infection and inflammation is complex but circulating inflammatory markers may not truly reflect the local inflammatory response in the lung. The aims of this study were to investigate the change of inflammatory biomarkers and cells within sputum and blood before and after intravenous antibiotics for a pulmonary exacerbation of CF. Methods: Assays included neutrophil elastase (NE) and complex, interleukin-8 (IL-8) and soluble intercellular adhesion molecule-1 (sICAM-1), fas ligand (FAS-L), and TNFr-1. Analysis of sputum cell differential and absolute cell counts and immunocytochemistry (CD11b and CD95) on sputum and isolated blood neutrophils were carried out. Results: There were no significant differences in absolute or differential sputum cell counts or sputum sol measurements following antibiotics. There was a significant increase in the percentage of blood neutrophils with minimal CD11b staining, 28 (4.1) mean percentage (SEM) versus 41 (2.9) and a decrease in the percentage showing maximal staining 30 (0.5) versus 15 (2.5). There was a significant increase in the percentage of blood neutrophils without CD95 staining, 43 (5.4) mean percentage versus 52 (5.1). Conclusion: These data suggest a modifiable systemic response to IV antibiotics but a local sustained inflammatory response in the lung.

Key words: cystic fibrosis (CF); neutrophil; inflammation; immunocytochemistry.

BACKGROUND

Chronic infection in cystic fibrosis (CF) and associated intermittent infective exacerbations with airway inflammation lead to progressive lung injury.1 Neutrophils are considered responsible for the onset and promotion of the inflammatory response in the CF lung.2 The mechanisms leading to this response remain largely unknown. It remains unclear whether infection precedes inflammation or vice versa. The relationship between infection and inflammation is complex. The host inflammatory response is not necessarily proportional to the burden of pathogens in the airways but the pathogens may trigger these responses.3 Lung inflammation is hard to quantify, therefore the systemic host inflammatory response is used as a marker of disease activity. It has been shown that inflammatory markers, C-reactive protein (CRP) and neutrophil elastase complex (NEC) fall significantly after a pulmonary exacerbation has been treated with intravenous (IV) antibiotics4 but circulating markers may not truly reflect the local inflammatory response in the lung. Resolution of this inflammatory process is not well understood. However, it is recognized that neutrophil apoptosis is an important mechanism for neutrophil removal at sites of lung inflammation, therefore limiting the potential proteolytic damage to the lung.5

The aims of this study were to investigate the modulation of soluble inflammatory factors within sputum, blood inflammatory markers, differential sputum cell counts, and neutrophil surface receptors before and after administration of IV antibiotics for a pulmonary exacerbation of CF. The differences in sputum sampling techniques were examined by comparing expectorated, physiotherapy, and induced sputum samples. We hypothesized that markers of inflammation would decrease and apoptosis increase following treatment.

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Grant sponsor: Respiratory Research Group, Belfast.

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Received 11 July 2006; Accepted 4 March 2007.

DOI 10.1002/ppul.20646
Published online 22 June 2007 in Wiley InterScience (www.interscience.wiley.com).

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MATERIALS AND METHODS

Sixteen patients with a pulmonary exacerbation of CF were admitted to the study. The diagnosis of CF had previously been established by genetic analysis and/or sweat testing. All patients had a sweat chloride concentration >60 mmol/L and one or two disease causing mutations. A pulmonary exacerbation was defined as an increase in pulmonary symptoms i.e., cough, sputum production, and shortness of breath and a ≥10% decrease in FEV₁ compared to baseline. Patients taking corticosteroids were excluded from the study. Patients were admitted to the Regional Adult Cystic Fibrosis Centre in the Belfast City Hospital (BCH) for 2 weeks of IV antibiotic therapy. Before the start of IV treatment (V1) and following completion of 14 days of treatment (V2), a number of parameters were assessed. This study was approved by the local ethics committee. Informed written consent was obtained in all cases.

Spirometry (FEV₁ and FVC) was carried out according to the American Thoracic Society guidelines. Patients were then asked to provide a spontaneously expectorated sputum sample (E). This was followed by a session of physiotherapy based on the active cycle of breathing; sputum samples were collected during and after this procedure and labeled (P). Finally sputum was induced using a methodology derived from Fahy and colleagues using 3% hypertonic saline and a Devilbiss 2000³ ultrasonic nebulizer (I). The three sputum samples collected were transferred immediately to the laboratory. Sputum plugs, free of salivary contamination were selected using plastic forceps (to a weight of 300 m) and placed in a sterile tube. The methodology used to process the sputum plugs was derived from Cai and co-workers. The remaining sputum was centrifuged at 30,000 g for 1 hr at 4°C. The sol was aspirated, protease inhibitors added, and frozen at −70°C for future analysis.

Free neutrophil elastase (NE) activity was measured in induced sputum sol by a colorimetric microtitre plate assay, using the elastase substrate Suc-Ala-Ala-Pro-Val-pNA (Bachem, Walden, Essex). The detection limit for the assay was 30 ng/ml. Intra-assay and inter-assay coefficients of variation were 4.5 and 8.2%, respectively.

Soluble neutrophil elastase complex (sNEC) was quantified in induced sputum sol by an enzyme-linked immunosorbent assay (ELISA). The lower detection limit for the assay was 5 ng/ml. Intra-assay and inter-assay coefficients of variation were 4.6 and 7.2%, respectively. The assay for plasma NEC (pNEC) was as above but with the following changes: the antibody pair used was sheep anti-human NE IgG and a peroxidase-conjugated sheep anti-human alpha-1-antitrypsin IgG (The Binding Site, Birmingham, England) and the plasma samples were diluted in PBS containing 0.05% (v/v) Tween-20.

Interleukin-8 (IL-8), soluble intercellular adhesion molecule-1 (sICAM-1), and soluble Fas ligand (sFAS-L) were measured using commercially available ELISA kits (R&D Systems, Abingham, Oxon, UK). The detection limits for the assays were 10 pg/ml, 0.35 ng/ml, and 3.6 pg/ml respectively.

Soluble TNF receptor-1 (sTNFR-1) was measured in sputum sol by sandwich ELISA. The assay had a detection limit of 0.5 ng/ml. Intra-assay and inter-assay coefficients of variation were 5.8 and 8.4%, respectively.

Blood samples included full blood count (FBC) and CRP, both measured by routine methods in NHS laboratories. Ear lobe blood gas analysis was carried out as follows. Transvesin³ cream was applied to one ear lobe for 10 min and then wiped off. Using a 21-gauge needle, the ear lobe was gently pricked to produce a small drop of blood. This blood was drawn into a heparinized glass capillary tube (Instrumentation Laboratory, Lexington, MA) via capillary action, by gently touching the drop of blood with one end of the tube. The capillary tube was immediately transferred to a blood gas analyzer Synthesis 10 (Instrumentation Laboratory). Neutrophils were isolated from peripheral blood using a method adapted from Firestein et al.¹⁴

Total cell counts and cell viability using the trypan blue exclusion test. Slides were prepared of blood and sputum neutrophil using the glass coverslip method as previously described.¹⁵ Differential cell counts were carried out on the slides using a Speedy-Diff (Clin-tech Ltd., Guildford, UK) kit according to the manufacturer’s instructions. The remaining slides were wrapped in aluminium foil and stored at −70°C for future analysis.

Cell surface receptor analysis was carried out as follows. Slides were removed from the −70°C freezer and left to thaw, still wrapped in aluminium foil, for 2 hr at room temperature. The slides were unwrapped and placed in a bath of 1% paraformaldehyde (PFA) for 5 min. Immunocytochemistry was performed using the DAKO APAAP Kit, System 40, K670 (Dako, Cambridgeshire, UK). The primary antibodies used were CD11b and CD 95 (Dako). The cells were then assessed for fast red staining (using a phase contrast microscope) i.e., absent (−), minimal (+), moderate (++) or maximal (+++) staining and counted (Fig. 1) as per previously described.¹⁶ All slides were assessed by one blinded observer.

Prism software was employed for the statistical analysis of this study. Differences before and after antibiotics were assessed using the paired Student’s t-test. Differences in cell counts between sputum methods were assessed using the Kruskal–Wallis test. Analyses on sol samples, CRP, pNEC, and total cell counts were carried out on log-transformed data. The transformations normalized the distributions. A P-value using two-tailed testing of <0.05 was considered statistically significant.
RESULTS

Patient Demographics

The mean age of the patients was 25 years, range (15–37). Sputum culture isolated *Pseudomonas aeruginosa* (Pa) alone from four patients, *Burkholderia cenocepacia* (Bc) from five patients, Pa and Bc from three patients, Pa and *S aureus*, Bc and *H influenzae*, *H influenzae*, and *S Pneumoniae* from one patient each. Antibiotic choice varied between patients, depending on microbiological susceptibility testing and clinical response. Initial antibiotic choices (two to three antibiotics per patient) included tobramycin (n = 14), ceftazidime (n = 10), aztreonam (n = 3), meropenem (n = 2), tazobactam (n = 2), gentamicin (n = 2), flucloxacillin (n = 1), and cefotaxime (n = 1).

Lung Function Tests and Blood Gases

Lung function tests (FEV1 and FVC) were compared at the start (V1) and end (V2) of IV antibiotics. Following completion of treatment, there was a significant increase in lung function. FEV1 1.88 (1.4–2.4) L mean (95% CI) versus 2.36 (1.7–3.0) L, \( P = 0.002 \), FVC 2.67 (2.2–3.1) L versus 2.97 (2.5–3.5) L, \( P = 0.01 \). Capillary blood gas analysis of pH, partial pressure of oxygen (pO2), partial pressure of carbon dioxide (pCO2), and oxygen saturation (O2 sat) showed no significant change after antibiotics (data not shown). There was a weak positive correlation between lung function and capillary oxygenation (pO2) before antibiotics (V1), FEV1 \( r^2 = 0.3, P = 0.04 \) and FVC \( r^2 = 0.3, P = 0.04 \), but this relationship was lost after completion of antibiotics (V2).

Blood Count and CRP

There was a small but statistically significant change in haemoglobin, 14.05 (0.31) g/dl mean (SEM) versus 13.62 (0.37) g/dl, \( P = 0.04 \). There was a statistically significant drop in CRP, 1.04 (0.16) mg/dl versus 0.62 (0.15), \( P = 0.03 \).

CELL COUNTS

Cell Viability

There was no significant difference in sputum cell viability between V1 and V2 for each method of sputum collection. (E) 80 (3.0)% versus 72 (5.1), \( P = 0.14 \), (P) 82 (3.1) versus 73 (4.0), \( P = 0.11 \), (I) 81 (2.8) versus 76 (3.7), \( P = 0.31 \). There were no significant differences in mean cell viability between each of the three sputum methodologies within V1 or V2.

Total Cell Counts

Sputum total cell counts (log-transformed data) were reduced after IV antibiotics and reached statistical significance in expectorated sputum. (E) 1.0(0.13) \( \times 10^6 \) cells/g sputum versus 0.48 (0.18), \( P = 0.04 \), (P) 1.09 (0.16) versus 0.91 (0.15), \( P = 0.4 \) and (I) 1.14 (0.14) versus 0.93 (0.13), \( P = 0.50 \) (Fig. 2). Differences in total cell counts between the three sputum methodologies were assessed with no significant differences observed within V1, \( P = 0.71 \), or V2, \( P = 0.14 \).

Percentage Cell Counts

Percentage neutrophils, macrophages, eosinophils, lymphocytes, and squamous cells were calculated. There was a non-significant decrease in (E) neutrophils,
94 (1.5)% versus 82 (6.8)%, \( P = 0.33 \) and increase in squamous cells 3 (0.8) versus 14 (6.3), \( P = 0.23 \) following antibiotics.

The differences in percentage cell type (neutrophils, macrophages, lymphocytes, eosinophils and squamous cells) between the three sputum methodologies at any one time point was assessed. There was a significantly greater percentage of squamous cells in expectorated sputum, 3 (0.8) compared with (P), 1 (0.3) and (I), 1 (0.8), \( P = 0.03 \) at V1.

**Absolute Cell Counts**

There were non-significant reductions in absolute neutrophil counts following antibiotics for each of the sputum methodologies, (E) 19 (5) \( \times 10^6 \) cells/g sputum versus 6 (4) \( P = 0.18 \), (P) 25 (6) versus 12 (4) \( P = 0.17 \), (I) 26 (6) versus 13 (5) \( P = 0.06 \). Comparing the cells between the three sputum methodologies at any one time point only the squamous cells differed significantly (E) 0.3 (0.07) \( \times 10^6 \) cells/g sputum compared to (P) 0.16 (0.06) \( \times 10^6 \) cells/g sputum and (I) 0.06 (0.02) \( \times 10^6 \) cells/g sputum, \( P = 0.03 \) at V1.

**Sputum Sol Measurements**

Sputum sol mediators (NE, sNEC, IL-8, sICAM-1, sFAS-L, and sTNFr-1) did not differ significantly after 2 weeks of antibiotic treatment (Table 1).

**Immunocytchemistry**

There was no significant change in levels of CD11b expression on sputum neutrophils following completion of IV antibiotic treatment. In contrast, there was a significant increase in the percentage of blood neutrophils exhibiting minimal CD11b staining and a significant decrease in the percentage showing maximal staining (Table 2). There was no significant change in levels of CD95 expression on sputum neutrophils following completion of IV antibiotic treatment. However, a significant increase in the percentage of blood neutrophils without CD95 staining was found and a trend for a decrease in the percentage showing maximal staining (Table 3).

**DISCUSSION**

A 2-week course of IV antibiotics for a pulmonary exacerbation of CF resulted in a clinical improvement which was reflected in a significant increase in lung function (FEV\(_1\) and FVC). We investigated the effects of this treatment on a selection of inflammatory biomarkers, differential cell counts, and neutrophil surface receptors. The differences in sputum recovery methods were also highlighted which has important practical implications.

Previous studies have examined differential cell counts in BAL and induced sputum in patients with CF and induced sputum has been used where expectoration was unsuccessful within the same study.\(^1\) This assumes the composition of expectorated and induced sputum to be similar. There have been no studies to determine that differing methods of obtaining sputum in patients with CF yield comparable total and differential cell counts. In this study, there was a trend for a greater number of cells in the physiotherapy and induced sputum per gram of sputum compared to the expectorated sputum, but this did not reach statistical significance. This difference may be due to improved cell recovery rather than true cell number increase. The expectorated samples tended to be more tenacious resulting in increased numbers of cells mixed with cellular debris being removed at the filtration stage. Regardless of the technique involved cell viability was similar in all groups.

Percentage cell counts were similar between groups but there was a significantly higher percentage of squamous cells in the expectorated compared to the induced sputum. Physiotherapy and induced sputum groups had higher absolute neutrophil and macrophage counts compared to expectorated sputum but this did not reach statistical significance. A possible explanation of cellular differences is that induced sputum may originate more distal in the airway than an expectorated sample. This area requires further investigation as changes in cellular composition between differing sputum samples suggest that induced

<table>
<thead>
<tr>
<th>NE (ug/ml)</th>
<th>V1</th>
<th>V2</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sNEC (ug/ml)</td>
<td>53.42 (30.5)</td>
<td>11.24 (3.06)</td>
<td>0.83</td>
</tr>
<tr>
<td>IL-8 (ug/ml)</td>
<td>4.61 (1.99)</td>
<td>1.41 (0.63)</td>
<td>0.33</td>
</tr>
<tr>
<td>sICAM-1 (ug/ml)</td>
<td>51.75 (5.74)</td>
<td>57.95 (12.6)</td>
<td>0.89</td>
</tr>
<tr>
<td>sFAS-L (ug/ml)</td>
<td>13.72 (2.16)</td>
<td>14.32 (2.98)</td>
<td>0.39</td>
</tr>
<tr>
<td>sTNFr-1 (ug/ml)</td>
<td>0.36 (0.1)</td>
<td>0.18 (0.01)</td>
<td>0.11</td>
</tr>
<tr>
<td>Data (( n = 16 )) are given as mean (SEM). ( P )-values were calculated on log-transformed data.</td>
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</table>
and expectorated samples should not be used interchangeably in studies.

It has been demonstrated that total cell counts in induced sputum are reproducible (within week) in stable patients with CF.20 Furthermore Watt et al. observed similar sputum total cell counts irrespective of the colonized pathogen (including Bc and Pa).21 Our investigation showed a reduction in sputum total cell counts following treatment for each of the sputum methodologies. This only reached statistical significance in the expectorated group, possibly due to the number of patients in the study. Percentage differential cell counts were similar following antibiotics; however a decrease was observed in expectorated sputum neutrophils with an increase in squamous cells. Difficulty in expectoration of sputum following antibiotics could account for this change.

A recent study has shown the reproducibility of sputum inflammatory markers in stable patients with CF.22 In our study, there was a non-statistically significant reduction in sputum sol biomarkers (NE and sNEC) following treatment. There was also a non-significant trend for reduction in WCC, PMN, and pNEC. CRP, which is a non-specific marker of inflammation, fell significantly after treatment. However, in a study by Hendry et al. significant reductions were observed in WCC, CRP, and pNEC following antibiotics.18

IL-8, a powerful neutrophil chemoattractant, did not change in our study. A recent investigation did show a reduction in IL-8 following IV antibiotics.23 Furthermore Ordonez et al. demonstrated a reduction in NE and IL-8 following IV antibiotics.24 This was in a larger cohort of younger patients who were given IV antibiotics for up to 34 days.

ICAM-1 contributes to neutrophil influx into the airway lumen and its expression is increased on inflamed endothelium by proinflammatory cytokines.25 Although the pathophysiological function of sICAM-1 remains to be specified, a shift of its lung concentration is likely to reflect increased cellular ICAM-1 expression.26 Following treatment with antibiotics there was no change in sICAM-1 in sputum sol despite a slight reduction in neutrophil counts, which may in part represent an ICAM-1-independent neutrophil recruitment pathway in CF lungs. However, Salva et al. showed that sICAM-1 levels in CF sputum were lower in those who were acutely ill compared to stable patients, with no change following IV antibiotics.27 The binding of sICAM-1 to CD11/CD18 receptor on neutrophils in acutely ill patients may explain this.27 Expression of sICAM-1 in CF sputum was lower in those who were acutely ill compared to stable patients, with no change following IV antibiotics.27

Expression of sputum neutrophil CD11b, an important ligand of ICAM-1 and a marker of neutrophil activation, did not significantly change following treatment. This suggests that unchanged expression of CD11b and sICAM-1 levels could reflect continued airway neutrophil activation. In comparison, blood neutrophil CD11b expression was reduced. This study in part supports our hypothesis that systemic inflammation is reduced following IV antibiotics, but there is a persistent inflammatory response in the airway. Thus, despite clinical improvement there is likely ongoing airway damage.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Percentage of Sputum and Blood Neutrophils With Absent (−), Minimal (+), Moderate (++), or Maximal (+++) CD11b Staining Before (V1) and After (V2) IV Antibiotics</th>
</tr>
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<tbody>
<tr>
<td>Sputum neutrophils</td>
<td>Blood neutrophils</td>
</tr>
<tr>
<td>V1</td>
<td>V2</td>
</tr>
<tr>
<td>−</td>
<td>21 (9.1)</td>
</tr>
<tr>
<td>+</td>
<td>17 (6.6)</td>
</tr>
<tr>
<td>++</td>
<td>30 (6.9)</td>
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<tr>
<td>+++</td>
<td>31 (10.5)</td>
</tr>
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</table>

Data (n = 16) are given as mean (SEM).
These effects should be further investigated with serial measurements of these markers in patients remote from a pulmonary infection.

We hypothesized that neutrophil apoptosis would be increased following treatment of a pulmonary exacerbation in order to help resolve the inflammatory process. CD95 or Fas is an important part of the cellular pathway regulating the induction of apoptosis. Its expression on sputum neutrophils did not alter with treatment but its expression on blood neutrophils decreased following antibiotics. sFAS-L is an inducer of apoptosis. Following treatment of pulmonary exacerbations, sFAS-L levels fell by half but did not reach statistical significance. This suggests that this pathway of apoptosis is more active at the beginning rather than the end of a pulmonary exacerbation disproving our hypothesis. But other apoptotic pathways may be involved. Soluble TNF-α was also reduced following treatment. Occupancy of TNF receptors by TNF-α initiates apoptosis; however prolonged incubation of neutrophils with TNF-α can reduce apoptosis.28 The soluble TNF receptor reflects TNF activation by inhibiting TNF action in vivo; it is therefore viewed as anti-inflammatory. Therefore within the complex inflammatory milieu in the CF lung, it is difficult to determine whether it functions as an inducer of neutrophil apoptosis or not. Measuring serial TNF-α levels, correlating with soluble receptors, and quantifying TNFα-1 with immunocytochemistry could help resolve this.

This study is the first to use immunocytochemistry of both sputum and blood neutrophils alongside soluble markers of neutrophil activation and apoptosis, to determine the effects of treatment on pulmonary exacerbations. The inflammatory process is complicated, although not reflecting the complete process, they do provide valuable information. Further work is required to explore these methods and ultimately improve our understanding of inflammation and its resolution within CF airways.

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