Cytokine concentrations and neutrophil elastase activity in bronchoalveolar lavage and induced sputum from patients with cystic fibrosis, mild asthma and healthy volunteers

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Abstract

Background: Induced sputum (IS) has been proposed as a non-invasive alternative to bronchoalveolar lavage (BAL) for the assessment and monitoring of airways inflammation. The aim of this study was to compare both methods in patients with cystic fibrosis (CF). The possible differences between subjects with CF, mild asthma and healthy volunteers (HV) was also assessed.

Method: In a single centre, randomised, two way crossover study, 11 patients with CF, 9 mild asthmatics (MA) and 11 HV underwent BAL and hypertonic saline induction on consecutive days. Free neutrophil elastase (NE), neutrophil elastase/α1-antitrypsin complex (NE–AAT), tumour necrosis factor receptor (p55) and interleukin-8 (IL-8) were measured in cell free supernatants.

Results: Three CF patients reported serious adverse events following BAL. NE was usually undetectable in both IS or BAL samples and NE–AAT concentrations did not differ consistently between the two sampling methods. IL-8 and p55 levels in the CF patients tended to be higher in IS samples compared with BAL samples (median 19 860 vs. 3855 pg/ml and 2.55 vs. 0.29 ng/ml, respectively). There was a significant difference in mean p55 concentrations between CF, MA and HV in IS samples (P < 0.003) but not in BAL samples (P = 0.36). The difference in mean IL-8 concentrations in IS samples between subject groups was statistically different (P = 0.023). Conclusions: IS samples can be safely obtained from CF patients. Analysis of IS samples can help to characterize the inflammatory process in the airways of CF patients. The serious adverse events following BAL in 3 CF patients highlight an inherent risk associated with this procedure.

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Keywords: Cystic fibrosis; Induced sputum; Lavage; Adverse event

1. Introduction

In patients with cystic fibrosis (CF) it is important to establish safe methods for the collection of airway specimens suitable for the assessment of disease activity and response to treatment. Not all patients with CF expectorate sputum spontaneously and certainly not in a reproducible manner. Bronchoalveolar lavage (BAL) has been used to sample airway fluid in CF patients and has enabled the measurement and evaluation of a range of proinflammatory mediators and cytokines considered important in the pathogenesis of injury in CF [1]. It is invasive and as sampling tends to be localised it may not reflect the inflammatory processes in the lung generally. Induced sputum (IS) has recently been studied in children with CF [2]. It appears a less invasive method to obtain samples, which may also better reflect the inflammatory processes in the airways [3].

It is unclear whether IS or BAL would be best suited for monitoring disease activity in patients with CF. The aim of this study was to compare these two methods of airway sampling in adult patients with CF.

2. Methods

2.1. Patient recruitment

Eleven patients with CF, 9 patients with mild asthma (MA) and 11 healthy volunteers (HV) were included in the study. All subjects were aged 18 years or older and had provided written informed consent. The study was
approved by the Research Ethics Committee of the Faculty of Medicine, The Queen’s University of Belfast.

2.1.1. Cystic fibrosis
The diagnosis was confirmed in childhood (sweat Na⁺ and Cl⁻ > 70 mmol/l) and all patients were chronically infected with Pseudomonas aeruginosa, Staphlococcus aureus, Haemophilus influenzae or Burkholderia cepacia and had mild impairment of lung function (FEV₁ > 70% of predicted value).

2.1.2. Mild asthma
Patients with a history of recurrent wheezing and dyspnoea with mild impairment of lung function (FEV₁ > 70% of predicted value).

2.1.3. Healthy volunteers
No evidence of clinically important medical disease and no history of upper respiratory tract infection in the preceding 2 weeks.

2.2. Exclusion criteria
CF patients and MA were excluded from the study if resting hypoxaemia (arterial oxygen saturation [SpO₂] < 92%) was evident on breathing room air or there had been an exacerbation of disease within the previous 30 days. All the CF and MA subjects were lifetime non-smokers and any HV who were current smokers or ex-smokers of <6 months were excluded from the study.

2.3. Study design
The design was a single centre randomised two way crossover study over 2 days with MA and HV serving as positive and negative controls, respectively. Following recruitment to the study, baseline assessment was performed with measurement of height and weight, together with obtaining venous blood samples for total white blood cell count and measurement of height and weight of pellet recorded. The supernatant was aliquoted and stored at −70 °C until assayed.

2.4. Bronchoscopy and bronchoalveolar lavage
All bronchoscopies were performed using a standard protocol [4]. BAL was performed (4×30 ml aliquots of sterile isotonic saline, right middle lobe). The returned samples were pooled, placed immediately on ice, taken to the laboratory and processed as described below.

2.5. Induced sputum
FEV₁ and forced vital capacity (FVC) were recorded using a vitagraph (Vitalograph, UK). Following this, two puffs (200 µg) of salbutamol were administered via a metered dose inhaler. Ten minutes later, sputum induction using 3% saline was performed using a DeVilbiss 2000® ultrasonic nebuliser with an output of 2.4 ml and an aerodynamic mass median diameter of 4.5 µm. After each period of nebulisation, spirometry was repeated and if the FEV₁ fell by more than 20% of the pre-saline value or the subject became symptomatic in any way the inhalation was terminated. All sputum produced during nebulisation was retained in a sterile container at room temperature.

2.6. Processing of samples
2.6.1. Bronchoalveolar lavage
An aliquot of the pooled BAL sample was removed for total cell count using an improved Neubauer haemocytometer and differential cell counts using the glass coverslip method [5]. The remaining BAL fluid was centrifuged (200×g, 10 min, 4 °C) after which the supernatant was removed and volume of supernatant and weight of pellet recorded. The supernatant was aliquoted and stored at −70 °C until assayed.

2.6.2. Induced sputum
Sputum was processed essentially using the method of Pavord et al. [6]. Sputum plugs were selected from the original known weight of IS, to minimise salivary contamination. The plugs were weighed, and 4 times the selected sputum volume of freshly prepared 0.1% (w/v) dithiothreitol (DTT) was added. After vortexing (15 s) and gentle mixing, samples were incubated in a shaking water bath at 37 °C for 15 min. Subsequently, a further 4 volumes of phosphate buffered saline (PBS) were added and incubated for a further 5 min. The resultant suspension was filtered through 53-µm gauze (Lockertex, Warrington, England), and centrifuged at 200×g for 10 min. The supernatant was removed and protease inhibitors added. For interleukin-8 (IL-8), neutrophil elastase/α₁-anti-trypsin complex (NE–AAT) complexes and p55 soluble TNF receptors, 3.7 mM EDTA (inhibiting metalloproteinase-like activity) (Sigma Chemicals, Poole, England), 150 µM TLCK (inhibiting trypsin-like activity) (Sigma Chemicals), 20 µM Z-Phe-diphenylphosphonate (inhibiting chymotrypsin-like activity) (supplied by the Biomolecular Sciences Group, School of Pharmacy, Queen’s University of Belfast) and 100 µM methoxy succinyl–Ala–Ala–Pro–Val–chloromethylketone (Sigma Chemicals) were added (final concentrations shown). For NE, all protease inhibitors, except methoxy succinyl–Ala–Ala–Pro–Val–chloromethylketone (NE inhibitor) were added in final concentrations as stated.
2.7. Assays

2.7.1. Free human neutrophil elastase

This was measured in IS or BAL using a kinetic, chromogenic microtitre plate assay using succinyl–Ala–Ala–Pro–Val–p–nitroanilide (Bachem) as a substrate. A standard curve, constructed from known concentrations of purified elastase, was included in each assay. Sputum or BAL samples were assayed at an initial dilution of 1:20 (100 μl) and the reaction initiated by the addition of substrate (100 μl of a 2 mg/ml solution gave a final concentration of 1 mg/ml). Hydrolysis of the substrate was monitored at 405 nm commenced immediately and at 1 min intervals for 40 min, during which the plate was maintained at 37 °C. The maximum change in OD per minute (V_{max/min}) was derived using BIOLISE software, and the corresponding elastase concentration was determined from the standard curve. The limit of quantification for the assay was 0.004 units/ml. Intra-assay and inter-assay coefficients were 4.5 and 8.2%, respectively.

2.7.2. Human neutrophil elastase complexed to α₁ antitrypsin

This was quantified by an enzyme-linked immunosorbent assay (ELISA). Briefly, microtitre plates were coated with anti-human neutrophil AAT IgG (The Binding Site, Birmingham, England) and the plate incubated overnight at 4 °C. Any unbound sites were blocked using a solution of 10% (w/v) Marvel (Premier Beverages, Adbaston, England) in PBS (200 μl/well) for 1 h at 37 °C. The plate was then washed briefly with PBS. A stock calibrant was prepared by mixing NE (Calbiochem, CN Biosciences UK, Nottingham, England) and α₁-antitrypsin (Calbiochem) to constitute a final concentration of NE–AAT complex of 20 μg/ml. The calibration curve was prepared by diluting the stock calibrant with 5% (w/v) Marvel in PBS to give a working solution of 4000 ng/ml. Fivefold serial dilutions were made until a concentration of 0.256 ng/ml was reached. Standards, IS and BAL samples were plated out in duplicate and incubated for 2 h at 37 °C. The NE–AAT complex was detected using a peroxidase-labelled sheep anti-human HE IgG (The Binding Site) for 2 h at 37 °C and a ready to use solution of 3,3′,5,5′-Tetramethylbenzidine (Calbiochem). Absorbance was measured at 450 nm on a SpectraThermo microtitre plate reader (Tecan, Reading, UK). Any sample duplicates with coefficient of variation greater than 10% were repeated. The detection limit for the assay was 5 ng/ml. Intra-assay and inter-assay coefficients of variation were 4.6 and 7.2%, respectively.

2.7.3. Interleukin-8

IL-8 levels were measured in sputum and BAL supernatant using a commercially available ELISA (R&D Systems, Abingham, Oxon, UK). The limit of quantification for the assay was 312 pg/ml. Intra-assay and inter-assay coefficients of variation were 5.6 and 7.4%, respectively.

2.7.4. p55 soluble TNF receptor

p55 receptors were measured by a sandwich ELISA [7]. Briefly, microtitre plates were coated with SR13 monoclonal antibody to soluble p55 overnight at room temperature. Blocking was achieved using 1% (w/v) bovine serum albumin. Samples and standards were incubated for 1 h, and p55 identified with biotinylated 5R5 monoclonal antibody in combination with streptavidin–peroxidase conjugate. Absorbance was measured at 450 nm. 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.

2.8. Statistics

Data are reported as median (range) unless otherwise stated. The BAL and sputum biochemical analyses were compared between the three groups using a one-way analysis of variance (ANOVA). Where an individual measurement was below the detection limit for the assay, the value was assumed to be equal to the detection limit when the data were summarised. Correlations were tested using Spearman’s rank method. A value of \( P < 0.05 \) was considered significant.

3. Results

3.1. Subject characteristics

Subject characteristics are summarized in Table 1. The bacterial pathogens cultured from sputum samples...
are displayed in Table 2. Ten of the 11 patients with CF had a history of infection with no organisms cultured from the asthmatic or healthy subjects. No significant difference in either total cell counts or neutrophil percentage in BAL was observed when *B. cepacia* positive and negative patients were compared. There was no significant correlation between percentage of neutrophils and lung function parameters (FEV1, FVC).

### 3.2. Free human elastase (NE)

Regardless of the method of collection, NE activity was below the limit of quantification in the majority of samples (11 of 19) from patients with CF. There was no clear pattern in the relative activities in samples collected by different methods. All BAL and IS samples collected from MA and HV had undetectable free elastase levels.

### 3.3. Elastase complexes (NE–AAT)

Concentration of NE–AAT complexes in samples collected by the two different methods from CF patients varied widely. However, the mean concentrations in both BAL and IS samples from patients with CF were greater than the corresponding samples from patients with MA or HV (Table 3).

### 3.4. p55 (TNFα receptor)

There was less variability in the p55 concentrations in IS and BAL samples from patients with CF. In CF patients, concentrations of p55 were consistently greater in IS samples compared to those obtained from BAL (median IS concentration, 2.55 ng/ml (range: 0.10–1.24 ng/ml) vs. median BAL concentration 0.29 ng/ml (range: 0.10–1.24 ng/ml)) (Fig. 1). Median concentrations in samples collected by IS differed significantly between patients with CF, patients with MA (1.74 ng/ml (range: 0.53–3.57 ng/ml)) and HV (0.20 ng/ml (range: 0.13–0.37 ng/ml)), *P* = 0.003, (Fig. 2). How-

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**Table 2**

Organisms isolated from sputum samples

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>8</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Haemolytic streptococcus</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3**

Concentrations of cells, E and AAT-E in samples obtained by bronchoscopy and IS from 11 CF patients, 9 mild asthmatic patients and 11 HV

<table>
<thead>
<tr>
<th></th>
<th>CF (<em>n</em> = 11)</th>
<th>MA (<em>n</em> = 9)</th>
<th>HV (<em>n</em> = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells × 10⁴ ml⁻¹</td>
<td>62.9 (14–113)</td>
<td>25.5 (6–83)</td>
<td>16.5 (10–23)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>43.7 (2.4–91.0)</td>
<td>59.1 (32.8–81.4)</td>
<td>86.6 (68.3–93.8)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>38.7 (1.5–96.5)</td>
<td>2.33 (0.2–7.4)</td>
<td>7.5 (0.6–25.1)</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>0.6 (0.1–9.0)</td>
<td>0.7 (0.1–1.3)</td>
<td>0.7 (0.25–0.9)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.18 (0–0.7)</td>
<td>4.3 (0–16.1)</td>
<td>0.5 (0–1.7)</td>
</tr>
<tr>
<td>Epithelial</td>
<td>4.95 (0.1–15.4)</td>
<td>29.5 (4.5–58.7)</td>
<td>4.7 (2.5–7.4)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>33.8 (17–50)</td>
<td>32.8 (21–50)</td>
<td>50.9 (33–75)</td>
</tr>
<tr>
<td><strong>IS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E (units/ml)</td>
<td>0.018 (0.004–0.023)</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>NE–AAT (μg/l)</td>
<td>0.281 (0.076–74.13)</td>
<td>0.08 (0.025–1.17)</td>
<td>0.381 (0.004–12.62)</td>
</tr>
<tr>
<td><strong>BAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E (units/ml)</td>
<td>0.004 (0.004–0.99)</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>NE–AAT (μg/l)</td>
<td>10.60 (0.001–10 000)</td>
<td>0.001 (0–0.056)</td>
<td>0.005 (0–0.009)</td>
</tr>
</tbody>
</table>

CF, cystic fibrosis; MA, mild asthmatic; HV, healthy volunteer; BAL, bronchoalveolar lavage; IS, induced sputum; E, free human neutrophil elastase activity; NE–AAT, neutrophil elastase/α₁-anti-trypsin complex. Values given as mean (range).
ever, no significant difference was noted in p55 concentrations between CF patients and BAL samples from MA and HV subjects. There was no correlation between p55 concentrations measured in BAL or IS from patients with CF (P = 0.584).

3.5. Interleukin-8

Among patients with CF, IL-8 concentrations tended to be greater in the IS samples than in the BAL samples (median IS concentration 19 860 pg/ml (range: 1439–59 847 pg/ml) vs. median BAL concentration 3855 pg/ml (range: <312–13 246 pg/ml)) (Fig. 1). The median IL-8 concentrations in IS from CF patients, patients with MA (5215 pg/ml (range: <713–14 155 pg/ml)) and HV (5379 pg/ml (range: <312–37 260 pg/ml)) differed significantly (P = 0.023, Fig. 3). IL-8 concentrations were undetectable in all the BAL samples from subjects with MA and HV. There was no between IL-8 concentrations measured in BAL or IS from patients with CF (r = 0.464, P = 0.302).

3.6. Differential cell counts

The differential cell counts in BAL for the three subject groups are shown in Table 2. As anticipated, neutrophil counts were significantly increased in the lavage specimens from CF patients compared with the MA and HV groups. Eosinophil numbers were increased in the MA group compared with CF and HV. There was no significant correlation between neutrophil percentages and IL-8 levels.

3.7. Adverse events

3.7.1. Induced sputum

All subjects tolerated the IS well. There was no significant reduction in FEV₁ during or following sputum induction in any of the subjects (data not shown).

3.7.2. Bronchoalveolar lavage

Adverse events were reported for 3 patients with CF, all resulting in prolonged hospitalisation.

Event 1: A 22-year-old male with CF who had B. cepacia isolated from sputum was enrolled in the study. At this time CRP was <1 mg/ml and WCC was 8×10⁹ cells/l. He was randomised to undergo IS on day 1, after which no adverse events were reported. He had BAL performed the following day during which he became hypoxic (SpO₂ = 82%). Shortly after he developed pyrexia and his temperature remained high for 5 days. WCC and CRP increased to 22×10⁹ cells/l and 152 mg/ml, respectively and took some weeks to normalise.

Event 2: A 31-year-old male with CF, who had Streptococcus pneumoniae isolated from sputum on the first day of the study, developed shortness of breath shortly after BAL. A CXR was performed, and demonstrated new changes of increased shadowing in the right mid zone. The symptoms persisted for 48 h.

Event 3: A 19-year-old male with CF, with B. cepacia and Pseudomonas aeruginosa previously isolated from sputum enrolled in the study and underwent IS without complication. However, 8 h after BAL he developed a severe pyrexia which lasted for 16 h and required an overnight stay in hospital.

4. Discussion

In this study we have demonstrated that biochemical markers of inflammation can be measured in IS samples from patients with CF. There was wide inter-patient variability in mediator levels within the different groups, possibly due to the relatively small number of subjects studied. IS appears to provide a sample more highly concentrated in inflammatory mediators than BAL. In addition, there were a number of serious adverse events.
following BAL highlighting the inherent risks associated with this procedure in CF patients.

Higher concentrations of IL-8 and p55 were consistently measured in the IS samples compared with BAL. It is likely that the technique of IS samples the proximal airways where most of the airway secretions are concentrated [3], whereas BAL fluid reflects events in the distal airways. Further dilution of cell and mediator levels by the saline lavage is likely to be greater than dilution caused by hypertonic saline during sputum induction. The demonstration of a more concentrated sample obtained with IS compared to that recovered at bronchoscopy has been reported in studies involving asthmatics and COPD patients [8,9]. However, as mediator concentrations were undetectable in the BAL samples from a number of the MA and healthy controls, our results need to be interpreted with caution.

Unfortunately, we are unable to report on cell differentials for the IS but based on work carried out in asthmatic [10] and COPD [9] populations we would anticipate that qualitative differences are likely to exist. IS, at least in asthmatics, is rich in eosinophils and neutrophil and poor in lymphocytes and macrophages compared with BAL [10]. As expected we found significantly more neutrophils in BAL from CF patients when compared with the healthy subjects and MA.

It is possible that the design of our study in which patients underwent BAL and IS on consecutive days may have influenced mediator and cell measurements or contributed to the adverse events. However, the aim of this study was not to quantify the effects of sampling methods on cell or mediator measurements. In any case, any effect is likely to have been minimised by the randomised design of the study. Furthermore, Fahy et al. have demonstrated that BAL sampling immediately after sputum induction appears safe and does not significantly influence cell and mediator levels measured in an isolated BAL sample 1 week earlier [8].

Bronchoscopy and BAL are generally well tolerated even when extended to asthmatics with severe disease [11]. Less is known about the safety of BAL in patients with CF. While IS appears to be safe when used for patients with severe airflow limitation, there is no data on the effects on CF patients. In this study, IS was safe and well tolerated by all patients. As recommended [12], pulmonary function was monitored frequently during sputum induction and no clinically or statistically significant reduction in FEV1 post sputum induction was noted. Adverse events were reported for 3 CF patients almost immediately following BAL. One patient became hypoxaemic during bronchoscopy and shortly after developed a severe pyrexia with an associated rise in CRP. A high fever lasting 16 h and requiring hospitalisation was also recorded in another CF patient and a further patient became dyspnoeic with right mid zone consolidation on CXR. In contrast, Konstan et al. reported on their BAL findings in clinically mild CF patients and observed a high fever (> 38.5°C), which they considered a minor reaction in only 2 of 18 subjects [1]. In our study, the CF patients had well preserved lung function. This is broadly representative of our stable CF population. We suspect that BAL may carry even greater risk in subjects with more severely impaired lung function.

Hypoxia during or following bronchoscopy is recognised especially in critically ill patients with compromised gas exchange [13]. Premedication may contribute to this and may have been the case with our patient although this does not explain the subsequent systemic response.

Fever has been reported to follow bronchoscopy and BAL in between 10 and 30% of cases and is usually considered a minor reaction [14]. The origin of the pyrexia is not known although an infectious mechanism seems possible. In one study of bronchoscopy and BAL, performed on ventilated patients, fever occurred in 73% of patients with pneumonia compared with only 17% in those without [15]. It is notable that only the CF patients had fever following BAL and as these patients have chronically infected lower airways, the lavage process may cause translocation of endotoxin into the circulation. Endotoxins are known to induce release of mediators such as TNFα and interleukins, which may be responsible for the fever [16]. In summary, IS is an acceptable technique for obtaining airway secretions from patients with CF. It is relatively non-invasive and when compared with BAL it may provide a richer source of the cells and mediators thought to play an important role in the pathophysiology of CF. Importantly, our observations suggest that even when performed on adult CF patients with stable disease, BAL appears a hazardous procedure which cannot be recommended in the research setting.

Acknowledgments

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References


