Comparison of spontaneous and induced sputum for investigation of airway inflammation in chronic obstructive pulmonary disease

A Bhowmik, T A R Seemungal, R J Sapsford, J L Devalia, J A Wedzicha

Abstract

Background—Although sputum induction is used as a technique to investigate lower airway inflammation in asthmatic subjects, advantages over spontaneous sputum in patients with chronic obstructive pulmonary disease (COPD) have not been investigated.

Methods—Samples of spontaneous sputum and sputum induced with 3% hypertonic saline for 14 minutes were collected from 27 patients with chronic obstructive pulmonary disease (COPD) who usually produced spontaneous sputum. Spirometric indices and oxygen saturation (SaO₂) were measured at seven minute intervals. The spontaneous, seven and 14 minute sputum samples were analysed for total and differential cell counts, cell viability, and interleukin 8 levels.

Results—Analysis of the sputum revealed that median cell viability was higher in the seven minute (62.8%; p = 0.004) and 14 minute (65%; p = 0.001) induced sputum samples than in spontaneous sputum (41.2%). There was no significant difference in total and differential cell counts or in interleukin 8 levels between spontaneous and induced sputum. During the sputum induction procedure the mean (SD) fall in forced expiratory volume in one second (FEV₁) was 0.098 (0.111) l (p < 0.001) and in forced vital capacity (FVC) was 0.247 (0.233) l (p < 0.001). There was a small but significant fall in SaO₂ during sputum induction (p = 0.03).

Conclusions—Induced sputum contains a higher proportion of viable cells than spontaneous sputum. There are no significant differences between the sputum samples obtained at seven minutes and at 14 minutes of hypertonic saline nebulisation. Sputum induction is safe and well tolerated in patients with COPD.

Methods

Patients
Twenty seven patients with a diagnosis of COPD who gave a history of daily sputum production volunteered to participate in this study. COPD was defined as forced expiratory volume in one second (FEV₁) less than 70% of predicted for age and height, and salbutamol reversibility less than 15% or 200 ml with airflow obstruction evidenced by an FEV₁/FVC ratio of <70%. Patients with a history of asthma, bronchiectasis, carcinoma of the bronchus, or other significant respiratory disease were excluded. Eight of the 27 patients were current smokers at the time of recruitment, a further 17 being former smokers. Twenty six of the 27 were on inhaled corticosteroids, one also being on oral steroids for arthritis. At recruitment baseline measurements were made of FEV₁, FVC, and peak flow by rolling seal spirometer (SensorMedics Ltd, Yorba Linda, California, USA), reversibility to salbutamol, and ear lobe capillary blood gas tensions (Model 278 Blood Gas Analyser, Ciba-Corning, Medfield, Massachusetts, USA).

The study was approved by the City and East London Health Authority research ethics committee and written informed consent was obtained from each patient.
COLLECTION OF SPONTANEOUS SPUTUM
Patients arrived at the clinic in the morning. Measurement of oxygen saturation (Minolta Pulsox 7, DeVilbiss Healthcare, Middlesex, UK) and spirometric tests were performed on arrival and repeated 10 minutes after premedication with 200 µg inhaled salbutamol via a multidose inhaler. Patients were instructed to blow their noses and rinse their mouths out with water before expectorating sputum into a sterile pot.

SPUTUM INDUCTION
Nebulisation with 3% saline was commenced using a procedure modified from that of Pin et al6 using the DeVilbiss UltraNeb2000 ultrasonic nebuliser. This nebuliser produced an aerosol output of approximately 2 ml/min with a mean particle size of 0.5–5 µm in diameter. After seven minutes of nebulisation measurement of oxygen saturation and spirometric tests were performed and nebulisation continued if the FEV1 had not fallen by more than 20%. After a further seven minutes the measurements were repeated and the procedure stopped. As before, patients were instructed to blow their noses and rinse their mouths out with water before expectorating sputum. Sputum samples collected during the first seven minutes and during the second seven minutes of the procedure were placed in separate sputum pots.

SPUTUM EXAMINATION
Sputum samples were examined as soon as possible, within two hours, adapting methods previously evaluated.1 6 7 The weight of the total sample was recorded. The sputum was then separated from contaminating saliva by macroscopic examination using a pair of disposable plastic forceps. The selected portion of the sputum was placed in a preweighed tube and the weight of the selected portion of the sputum recorded. The sputum was then mixed with four times its weight of freshly prepared 0.1% dithiothreitol solution made by mixing dithiothreitol powder with Hank’s balanced salt solution (HBSS). This was vortexed for 15 seconds, with water before expectorating sputum into a sterile pot. The suspension was then filtered through 50 µm nylon gauze to remove mucus and debris without removing any of the cells and centrifuged at 790g (2000 rpm) for 10 minutes. This resulted in the formation of a cell pellet and a supernatant solution. The supernatant was decanted off and stored at –70°C for future analysis and the cell pellet was resuspended in 400–1200 µl (depending on macroscopic estimation of the size of the cell pellet) of HBSS. The total cell count was determined with a Neubauer haemocytometer using the trypan blue exclusion method to determine cell viability; blue cells being counted as non-viable. The absolute number of non-squamous cells per gram of the original sputum sample was determined and the percentage of viable and non-viable cells obtained. The cell suspension was then mixed with HBSS to obtain a count of 0.6–1.0 × 10⁶ cells/ml of the suspension. Cytospins were then made using a Cytotek cytocentrifuge. The cytospin slides were stained with DiffQuik to obtain differential cell counts made by counting 400 cells per slide. Levels of IL-8 in the supernatant samples were measured using a quantitative sandwich immunnoassay. A monoclonal antibody specific for the cytokine was precoated onto a microtitre plate and standards and samples added. After washing, an enzyme linked polyclonal antibody was added followed by a substrate solution for colour development and intensity reading (R&D Systems Europe, Abingdon, Oxon, UK).

Table 1  Mean (SD) baseline physiological characteristics of patients with COPD

<table>
<thead>
<tr>
<th>Number</th>
<th>Age (years)</th>
<th>FEV1 (l)</th>
<th>FEV1 (%)</th>
<th>FVC (l)</th>
<th>FVC (%)</th>
<th>SaO2 (%)</th>
<th>PaO2 (kPa)</th>
<th>PaCO2 (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 (21M, 6F)</td>
<td>66.3 (8.1)</td>
<td>0.95 (0.37)</td>
<td>38.2 (14.6)</td>
<td>2.11 (0.62)</td>
<td>94.08 (2.91)</td>
<td>8.61 (1.02)</td>
<td>5.99 (0.98)</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis
Spirometric results and blood gas tensions are expressed as means and standard deviations. Data pertaining to sputum are expressed as medians and ranges. The Wilcoxon signed rank test was used to compare the cell viability in the spontaneous, seven minute and 14 minute sputum samples and paired t test to compare the physiological measurement data for the different points in time. The statistical package SPSS was used for data analysis.

Results
Baseline characteristics of the patients are shown in table 1. The mean (SD) FEV1 was 0.95 (0.37) l. The mean (SD) FEV1/FVC ratio was 45.3 (12.9) %.

The median weights of total (and selected) sputum obtained spontaneously and at seven and 14 minutes were 1.6 (0.4) g, 4.0 (0.6) g, and 4.1 (0.5) g, respectively. The sputum samples analysed had a median squamous cell contamination of 6.4% in spontaneous sputum (12 samples containing more than 10%), 6.0% in induced sputum at seven minutes (eight samples containing more than 10%), and 4.6% in induced sputum at 14 minutes (eight samples containing more than 10%). The volumes obtained were, however, variable.

Significantly higher cell viability was obtained in induced sputum than in spontaneous sputum (table 2). The median viability was 41.2% in spontaneous sputum, 62.8% in induced sputum at seven minutes, and 65% in induced sputum at 14 minutes. The difference between the median viabilities in spontaneous and seven minute sputum samples was 21.6% (p = 0.004), and between spontaneous and 14 minute sputum samples was 23.8% (p = 0.001). Differential counts could be performed on 18 of the 25 spontaneous sputum samples, 23 of the 27 sputum samples obtained at seven minutes, and 22 of the 25 sputum samples obtained at 14 minutes. Counts could not be
Table 2 Comparison of cell counts, cell viability, and interleukin 8 levels in spontaneous and induced sputum samples (n = 27)

<table>
<thead>
<tr>
<th>Median (interquartile range)</th>
<th>Spontaneous sputum</th>
<th>Induced sputum at 7 minutes</th>
<th>Induced sputum at 14 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total counts x 10^6 cells/g</td>
<td>3.43 (1.66–7.17)</td>
<td>1.91 (0.76–5.15)</td>
<td>2.10 (1.31–5.91)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>88.0 (72.5–89.5)</td>
<td>88.0 (70.0–93.5)</td>
<td>81.3 (77.8–91.8)</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>11.0 (6.3–23.8)</td>
<td>10.3 (4.8–22.5)</td>
<td>9.0 (6.5–18.3)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>1.5 (0.8–3.8)</td>
<td>2.0 (0.5–5.3)</td>
<td>2.0 (1.0–2.5)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.3 (0.3–0.5)</td>
<td>0.3 (0.0–1.3)</td>
<td>0.5 (0.0–0.8)</td>
</tr>
<tr>
<td>Cell viability (%)</td>
<td>41.2 (11.7–71.8)</td>
<td>62.8 (35.5–76.5)*</td>
<td>65.0 (54.7–76.2)*</td>
</tr>
<tr>
<td>Interleukin 8 (pg/ml)</td>
<td>5475 (1265–9083)</td>
<td>4288 (2653–6282)</td>
<td>3847 (747–8539)</td>
</tr>
</tbody>
</table>

*p = 0.001 compared with spontaneous sputum sample.

**Discussion**

This study showed that cell viability was significantly higher in induced sputum than in spontaneous sputum samples and there was no significant difference in total and differential cell counts and levels of IL-8 between the different sputum samples. The technique of sputum induction was shown to be well tolerated in patients with COPD.

With increasing interest in the mechanisms of inflammation in patients with COPD, there is a need to obtain airway samples by a convenient and non-invasive technique. Sixty two percent of patients with COPD produce daily spontaneous sputum, but it is important that all patients with COPD, whether or not they produce spontaneous sputum, are studied with a standardised clinical sample. Patients with severe COPD are unable to tolerate bronchoscopy. There are particular difficulties in evaluating patients who are suffering from exacerbations and in whom we need information about airway inflammatory changes. The procedure has potential as an investigatory tool in patients who do not produce sputum spontaneously. Sputum induction is thus particularly useful in obtaining clinical samples from these patients in order to provide specific information on cellular and molecular aspects of airway inflammation. These samples are also more concentrated and richer in airway secretions than those obtained by bronchoscopy.

This study showed that there is a large variability in the total cell counts and this may reflect the variability in the physical properties of the sputum. Whereas some samples contained large amounts of tenacious mucus, much of which had to be filtered, other samples were more fluid and easily homogenised, leading to better cell recovery. The higher proportion of viable cells found in induced sputum may be explained by the fact that sputum induction causes the mobilisation of a newer cell population after an older, and perhaps dying, population of cells has been spontaneously expectorated.

As previously shown, there was a relatively high proportion of neutrophils in the lower airway samples of patients with COPD which suggests that neutrophilic inflammation is the major factor in the pathology of the disease. Sînăscu et al have shown that airways obstruction and chronic expectoration, as well as rapid decline in FEV₁, are associated with increased numbers of neutrophils in the sputum. The sputum induction procedure itself is considered a stimulus for the appearance of greater numbers of neutrophils in the

Figure 1 Changes in FEV₁ and FVC at start of procedure and after seven and 14 minutes of sputum induction.
sputum, but these effects are seen some hours after nebulisation.14 We have not taken repeated sputum samples within the space of a few hours but an interval of at least one week between nebulisations is desirable to achieve meaningful results.15 Eosinophil and lymphocyte counts were low but variable in individual patients. A drawback of the technique of sputum analysis can be the relative difficulty in accurate recognition of lymphocytes on cytospin preparations.5 We found a difficulty in accurate recognition of lymphocytes on cytospin preparations.5 We found no differences in the differential counts between spontaneous and induced sputum and the results from induced sputum at seven and 14 minutes were similar. It may be possible, therefore, to stop sputum induction when an appropriate volume of sample has been obtained without the need to carry on for the full period. Another recent study16 has, however, found significant differences in these counts. This may be partly due to the fact that this particular study looked at normal and asthmatic subjects rather than patients with COPD, and the sputum induction procedure was conducted for significantly longer and with higher concentrations of hypertonic saline in the latter stages.

Dithiothreitol breaks up disulphide bonds, which are present in IL-8, and may thus theoretically interfere with the assay.7 We found that, as other work has shown, IL-8 measurements were not affected by the presence of dithiothreitol in the sputum supernatants at the concentrations used here.7 Higher concentrations of dithiothreitol used by some groups affect the concentration of IL-8 and would require an adjustment when calculating levels. In such a case an alternative method of sputum processing has been used with ultracentrifugation to separate the sol phase of the sputum from the cells.2

The procedure of sputum induction in this group of patients was well tolerated, although three of the patients experienced symptoms and there was an asymptomatic fall in FEV1 of just over 20% in another. Induced sputum was also relatively easy to obtain in these patients with COPD compared with asthmatic subjects,14 14 minutes of nebulisation producing an adequate sample in most cases.

This study shows that induced sputum is useful in practice as a method of providing standardised lower airway samples in patients with COPD, especially for clinical investigations. With the technique, much valuable information will be obtained about airway inflammatory changes and response to treatment without requiring invasive procedures.

The authors thank DeVilbiss UK for the loan of the Ultraneb 2000 nebuliser used in this study.

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