Long-term Repeatability of Induced Sputum Cells and Inflammatory Markers in Stable, Moderately Severe COPD*

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Study objectives: Neutrophilic inflammation is a major feature of COPD. Induced sputum is increasingly used to monitor inflammatory airway diseases. Although short-term repeatability of selected sputum markers has been extensively studied in several populations, data on the long-term repeatability of induced sputum markers in stable COPD are scant.

Design: Sputum supernatant of 12 patients with stable COPD was analyzed on three separate occasions with 4-weekly intervals. Sputum cells and inflammatory markers interleukin (IL)-8 and soluble intercellular adhesion molecule (sICAM)-1 were measured in supernatant using enzyme-linked immunosorbent assay. Repeatability of sputum markers was expressed by intraclass correlation coefficients (Ri).

Measurements and results: Sputum induction was safe in all patients. None of the sputum parameters analyzed changed significantly throughout the study. The repeatability for cell differential counts in stable COPD was as follows: total cells, Ri = 0.07; neutrophils, Ri = 0.66; macrophages, Ri = 0.47; eosinophils, Ri = 0.49; and lymphocytes, Ri = 0.58. The repeatability of soluble markers was as follows: IL-8, Ri = 0.50; and sICAM, Ri = 0.58. Sputum neutrophils were negatively correlated with lung function on each separate occasion, whereas soluble markers were not correlated with sputum cells (p > 0.16, all correlations) or lung function (p > 0.24, all correlations).

Conclusions: Clinically stable, moderate COPD is associated with equally stable sputum inflammatory markers. Repeatability of induced-sputum markers of neutrophilic inflammation in stable COPD is satisfactory, even over extended periods of time. These data support the usefulness of serial monitoring of induced-sputum inflammatory markers in COPD.

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Key words: COPD; induced sputum; interleukin-8; neutrophils; repeatability; soluble intercellular adhesion molecule

Abbreviations: FEV₁/FVC ratio; IL = interleukin; sICAM = soluble intercellular adhesion molecule-1

COPD is a debilitating inflammatory disease of the lungs characterized by an increased presence of neutrophils and macrophages in the airways of affected patients.¹,² Neutrophils produce proinflammatory mediators, cytokines, and proteases.³,⁴ Recruitment of neutrophils to the airways involves chemotactic stimuli such as interleukin (IL)-8 and adhesion of neutrophils to pulmonary and bronchial epithelial cells with subsequent migration into the airways and alveoli by integrin-dependent mechanisms.⁵,⁶ Of these, binding of neutrophil CD11b/CD18 (Mac-1) to intercellular adhesion molecule-1 is of pivotal importance. Soluble intercellular adhesion molecule-1 (sICAM-1) has been shown to be elevated in bronchial secretions and BAL in patients with predominantly neutrophilic airway inflammation.⁷,⁸

Induced sputum is a safe and noninvasive method to investigate inflammatory airway diseases such as asthma or COPD. Its use for diagnostic or scientific purposes has increased dramatically over recent years. Measurements of cellular composition are reliable, valid, and responsive to changing conditions.⁹–¹⁴ Moreover, a growing number of cytokines or mediators have been quantified in induced sputum. Whereas some among them are valid and reproducible,¹⁵,¹⁶ others show poor results.¹⁷...
reasons for this often being speculative. With this often poor validity and repeatability in mind, several authors have indeed recommended that physicians be critical about monitoring sputum mediators routinely or in clinical trials.18

Most trials studying the repeatability of induced sputum have focused on short-term repeatability, ie, measurements within days, or between 2 days and 3 days, and these studies were often carried out in healthy subjects or mild asthmatics. However, long-term repeatability of induced sputum may be of greater relevance to everyday clinical practice as well as to clinical trials, and few articles have addressed this particular issue. Therefore, the present study investigates the long-term repeatability of induced-sputum markers of neutrophilic inflammation in stable COPD. In our analysis, we will show for the first time, that repeated measurements of sputum markers are reproducible in stable, moderate COPD over a period of 8 weeks.

**Materials and Methods**

**Patients**

Twelve patients with diagnosed COPD were included into the analysis. All patients fulfilled diagnostic criteria of the Global Initiative for Obstructive Lung Disease for stage 2 (moderate) COPD.19 All patients had negative skin-prick test results to standard aeroallergens, a smoking history of at least 10 pack-years, FEV1 values between 40% and 70% of predicted, a FEV1/FVC ratio (FEV1/FVC%) of < 70%, and a postbronchodilator reversibility of FEV1 < 12%, measured at baseline 15 min after inhalation of a β-agonist (salbutamol, 400 μg via metered-dose inhaler). None of the patients had actively smoked for at least 6 months prior to the study; received inhaled or oral corticosteroids or other systemic drugs, including theophylline, antibiotics, or nonsteroidal anti-inflammatory drugs; or reported any acute exacerbations for at least 4 weeks prior to sputum induction. Only COPD patients without concomitant inhaled or oral corticosteroids for at least 3 months before baseline sputum induction were included in the analysis, since corticosteroids may influence cytokine levels,20,21 or neutrophil activation,22 although they have little or no effect on neutrophilic inflammation.10,21,24 The study was approved by local regulatory authorities, and each patient gave informed written consent.

**Pulmonary Function Tests**

Spirometry was performed using the JAEGER Masterscope spirometry system (Jaeger; Wuerzburg, Germany). The best of three consecutive spirometry recordings were used, following American Thoracic Society guidelines.25 Measurements included FVC and FEV1. All FEV1 values represent the prebronchodilator values.

**Timing of Sputum Induction**

All patients under survey were participants in a randomized, placebo-controlled, crossover study of 4 weeks of treatment with an investigational anti-inflammatory drug. Crossover treatment periods were separated by a 4-week washout period. There was a sputum induction before and after each treatment period. In patients receiving placebo during the first study period, sputum collected at baseline, after 4 weeks of placebo treatment, and finally after 4 weeks of washout was analyzed. In patients receiving placebo in the second study period, baseline sputum was obtained after the 4-week washout period, after placebo treatment, and on an additional visit 4 weeks after the end of the trial. This resulted in a total of three consecutive sputum inductions in 4-weekly intervals for each individual patient.

**Sputum Induction**

Sputum induction was performed according to a method previously described. Briefly, patients received two puffs of salbutamol (100 µg/puff) 15 min prior to the procedure, and then inhaled 3% hypertonic saline solution delivered by an ultrasonic nebulizer device (DeVilbiss; Hounslow, UK) for 15 min. Patients were told to rinse their mouth, blow their nose, and carefully cough sputum into a Petri dish using forced expiratory maneuvers. The first portion of sputum was discarded, and the inhalation procedure was continued for further 15 min. Lung function was carefully monitored by spirometry every 5 min during induction to ascertain safety of the procedure. Induction was stopped when the total length of induction of 30 min was completed or a > 15% drop in FEV1 occurred. In the latter scenario, patients were administered two further puffs of salbutamol.

**Sputum Processing and Counting**

Sputum plugs were closely examined by light microscopy to ascertain least possible contamination of sputum with squamous cells. An appropriate sample was then placed into a 1-mL Eppendorf tube, weighed, and mixed with the corresponding volume of 0.1% dithiothreitol (Calbiochem; Bad Soden, Germany) in phosphate-buffered saline solution (Gibco Life; Paisley, Scotland), as proposed by Pizzichini et al.26 Sputum was gently vortex mixed and placed into a water bath at 37°C for 15 min to allow homogenization of the sample. This procedure diluted the sputum and concentration of dithiothreitol twofold. Samples were centrifuged (2,800 revolutions per minute for 10 min), the supernatant was aspirated, and sputum cells were counted after cytopsin preparation and staining with Hemacolor-staining (Merck; Oarmstadt, Germany) to assess the quality of the sample. Finally, supernatant was recentrifuged (3,000 revolutions per minute for 5 min) to completely remove cellular components, and immediately frozen at −70°C. Only supernatant of sputum samples with a squamous cell contamination of < 20% was used for further analysis.

**Quantification of Soluble Factors in Sputum Supernatant**

The concentrations of IL-8 and sICAM in sputum supernatant were measured by commercially available immunoassays (IL-8: Pharmingen; Heidelberg, Germany; sICAM: BenderMedSystems; Vienna, Austria). Sputum supernatants were further diluted for the IL-8 assay (100-fold dilution). The lower detection limits of the assays were 1 pg/mL (IL-8) and 0.625 ng/mL (sICAM).

**Statistical Analysis**

Statistical analysis was performed using the STATA 5.0 software package (Stata Corporation; College Station, TX). Data are
presented as mean values ± SD. Parameters were tested for normal distribution using the Kolmogorov-Smirnov test. Repeated analysis of variance was used for paired mean data comparisons between visits. Repeatability of sputum measurements on three occasions was determined by using intraclass correlation coefficient (Ri). Representative examples of repeatability (IL-8 x sICAM) between individual visits were graphically reported as proposed by Bland and Altman,28 where the limits of agreement are expressed as ±2 SD of the mean of differences between two measurements within which 95% of the differences of repeated measurements are expected to be. Correlations were calculated by simple linear regression after log transformation of log normally distributed values (IL-8, sICAM). A p value < 0.05 was considered statistically significant.

RESULTS

Sputum Cells

All patients were able to produce adequate sputum samples on each occasion. During the whole study period, there was no significant change in total sputum cells, cell differentials (percentage), or absolute cell counts per milliliter of sputum. The reproducibility of sputum cell differentials was generally acceptable with Ri values > 0.47. However, total sputum cells were poorly reproducible (Ri = 0.07), as well as the total neutrophil and eosinophil count per milliliter of sputum (Ri = 0.33 and 0.19, respectively) [Table 1].

Correlation of Sputum Cells With Lung Function

On all three visits, the percentage of sputum neutrophils was inversely correlated with the extent of airflow limitation, expressed as the FEV1/FVC. The individual correlations were as follows: r = −0.69 (visit 1, p = 0.013), r = −0.59 (visit 2, p = 0.04), and r = −0.53 (visit 3, p = 0.07). The overall correlation was r = −0.58, p = 0.0002 (Fig 1).

Table 1—Reproducibility of Spirometry and Induced-Sputum Parameters Measured at 4-Weekly Intervals*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Visit 1</th>
<th>Visit 2</th>
<th>Visit 3</th>
<th>Difference Between Visits, p Values</th>
<th>Ri</th>
<th>Ri, p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1, % predicted</td>
<td>58 ± 7</td>
<td>56.9 ± 8</td>
<td>58 ± 12</td>
<td>&gt; 0.09</td>
<td>0.83</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>57.1 ± 10.5</td>
<td>56.5 ± 11.4</td>
<td>56.6 ± 12.6</td>
<td>&gt; 0.59</td>
<td>0.94</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Total cells, ×10⁶/mL</td>
<td>4.9 ± 2.7</td>
<td>4.9 ± 3.6</td>
<td>4.7 ± 3.0</td>
<td>&gt; 0.84</td>
<td>0.59</td>
<td>0.07</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>57 ± 20</td>
<td>55 ± 27</td>
<td>63 ± 18</td>
<td>&gt; 0.14</td>
<td>0.66</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Neutrophils, ×10⁶/mL</td>
<td>2.8 ± 1.9</td>
<td>3.3 ± 2.9</td>
<td>3.3 ± 2.5</td>
<td>&gt; 0.47</td>
<td>0.32</td>
<td>0.034</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>27 ± 15</td>
<td>36 ± 20</td>
<td>26 ± 13</td>
<td>&gt; 0.06</td>
<td>0.47</td>
<td>0.004</td>
</tr>
<tr>
<td>Macrophages, ×10⁶/mL</td>
<td>1.4 ± 1.3</td>
<td>1.24 ± 0.8</td>
<td>1.33 ± 0.7</td>
<td>&gt; 0.71</td>
<td>0.47</td>
<td>0.005</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>2.2 ± 2.7</td>
<td>1.5 ± 3.7</td>
<td>1.2 ± 1.7</td>
<td>&gt; 0.08</td>
<td>0.49</td>
<td>0.0026</td>
</tr>
<tr>
<td>Eosinophils, ×10⁶/mL</td>
<td>0.15 ± 0.19</td>
<td>0.34 ± 0.92</td>
<td>0.1 ± 0.16</td>
<td>&gt; 0.29</td>
<td>0.13</td>
<td>0.22</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>6.4 ± 3.9</td>
<td>5.8 ± 5.4</td>
<td>5.2 ± 3.5</td>
<td>&gt; 0.24</td>
<td>0.58</td>
<td>0.0004</td>
</tr>
<tr>
<td>Lymphocytes, ×10⁶/mL</td>
<td>0.35 ± 0.32</td>
<td>0.27 ± 0.28</td>
<td>0.27 ± 0.2</td>
<td>&gt; 0.30</td>
<td>0.47</td>
<td>0.004</td>
</tr>
<tr>
<td>Log IL-8, ng/mL</td>
<td>2.4 ± 1</td>
<td>2.2 ± 1.5</td>
<td>2.4 ± 0.9</td>
<td>&gt; 0.64</td>
<td>0.50</td>
<td>0.0033</td>
</tr>
<tr>
<td>Log ratio IL-8/cells</td>
<td>0.67 ± 0.98</td>
<td>1.2 ± 1.27</td>
<td>0.71 ± 0.79</td>
<td>&gt; 0.07</td>
<td>0.57</td>
<td>0.0005</td>
</tr>
<tr>
<td>Log ratio sICAM/cells</td>
<td>2.6 ± 1.9</td>
<td>3.8 ± 2.2</td>
<td>3.1 ± 1.8</td>
<td>&gt; 0.16</td>
<td>0.58</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD. Ri is the intraclass correlation coefficient for three individual measurements in 12 patients. p Values of differences of mean values between visits were calculated by repeated analysis of variance and represent the lowest p value for each comparison.

Figure 1. Correlation of sputum neutrophils (percentage) with the degree of airflow limitation, expressed as the FEV1/FVC, on separate visits. Open circles = visit 1, r = −0.69, p = 0.013; closed squares = visit 2, r = −0.59, p = 0.04; closed triangles = visit 3, r = −0.53, p = 0.07. The overall correlation for all visits was r = −0.58 (p = 0.0002).

Soluble Factors

Repeatability of soluble sputum markers is given in Table 1. In general, there were no differences in mean values between visits. Satisfactory repeatability was observed with both log sICAM (Ri = 0.58), and log IL-8 levels (Ri = 0.50) [Fig 2]. Repeatability of IL-8 further improved, when total IL-8 levels were divided by the number of total sputum cells (log ratio IL-8 ng/cells × 10⁶/mL).

Correlation of Soluble Factors With Sputum Cells and Lung Function

On individual visits, there was no correlation of any soluble sputum marker with cellular or spirometric
parameters. In particular, neither IL-8 nor sICAM correlated with lung function ($p > 0.24$, both correlations), sputum neutrophils ($p > 0.16$, both correlations), or total sputum cells ($p > 0.4$, both correlations) on any of the three visits. However, taking all three visits together, there was a significant correlation of log IL-8 levels with total sputum cells ($r = 0.48$, $p = 0.004$).

**Safety of Sputum Induction**

Sputum induction was safe in all patients. The mean drop in $\text{FEV}_1$ for all inductions was $-11.5 \pm 10.3\%$ change from baseline, with only four patients experiencing a drop in $\text{FEV}_1$ of $>20\%$ after the final induction period. These four patients recovered completely after inhalation of another two puffs of salbutamol. All patients were able to adhere to the induction protocol and produced adequate sputum samples.

**DISCUSSION**

Neutrophilic inflammation is an important feature of COPD, and targeting the neutrophil is regarded as a potential future treatment for COPD. The present study was designed to assess the repeatability of sputum cells and markers of neutrophilic inflammation by repeated measurements over a time period of 8 weeks. First, our observations clearly demonstrate that clinical stability in moderate COPD is also reflected by the “stability” of surrogate markers in induced sputum. None of the parameters studied revealed any significant change over a study period of 8 weeks. From a methodologic point of view, our results further indicate that the majority of sputum factors studied in our analysis is sufficiently reproducible to warrant serial assessment in clinical routine or for research purposes.

There is wide agreement that $R_i$ values $>0.6$ represent a clinically acceptable degree of repeatability, when two measurements at different time points are analyzed. However, with repeated measurements over time, the acceptable limits may be lower, and other authors have used $R_i$ values of 0.4 to 0.5 as an acceptable limit. Taking into account the relatively small number of patients in our analysis and the fact that three measurements were obtained over 8 weeks, cell differentials of induced sputum were sufficiently reproducible over time. This observation stands in line with several other reports on short-term repeatability of sputum cells in asthmatics or healthy individuals. Good reproducibility was also observed for sputum lymphocytes in our patients, although their repeatability in induced sputum of selected patient populations was judged to be poor by other authors. The reasons for these discrepancies remain speculative. A possible explanation could be the adherence to varying induction lengths in most of the articles cited above. It has been repeatedly shown that varying induction times lead to changes in cellular and soluble components of induced sputum, and samples derive from more peripheral airways with rising induction length. At least in two of the articles reporting poor repeatability of sputum lym-
phocytes, there was no strict adherence to induction time. However, in the same articles, other sputum parameters were perfectly reproducible; therefore, the true reasons for the observed discrepancy remain elusive.

In agreement with other investigations, the repeatability of sputum cell differentials was unaffected by the poor repeatability of total cells in our study population. However, the latter most likely accounted for the discrepancies of repeatability between percentage and total cell count of sputum neutrophils and eosinophils. Moreover, this variability has to be taken into account when analyzing soluble markers in induced sputum, since many sputum proteins can be directly produced or secreted by sputum cells. Several studies have found a correlation of sputum IL-8 and sputum neutrophils in asthma and COPD. Given the fact that IL-8 is a major chemoattractant for neutrophils, this observation seems reasonable at first glance. However, it should be taken into account that neutrophils among other cells produce IL-8; therefore, the observed correlation may as well be a rather noncausal or merely associated phenomenon. This consideration is at least partially supported by data from our study, since there was an overall correlation of sputum total cells with IL-8, and the repeatability of IL-8 between visits improved when IL-8 levels were corrected for total sputum cells. Finding underlines the imminent need to compare indexes of cytokines or mediators relating to total sputum cells with pure concentrations in future studies.

Nevertheless, there was no correlation of sputum IL-8 and neutrophils in our population, regarding either individual visits or pooled data from all visits. Although this lack of association may be a consequence of the relatively small number of patients in our study, a strong correlation would have clearly derived from our study, since there was an overall correlation of sputum cell differentials being unaffected by induction length, safety of sputum induction becomes an important issue, since the required induction length may not be tolerated by all patients, especially those with severe airway obstruction. In our study, nearly all patients were able to adhere to induction times, although FEV1 declined significantly in selected patients. However, taking into account the grade of COPD severity (Global Initiative for Obstructive Lung Disease stage 2) in our patients, it can be concluded that sputum induction was safe in this population.

In summary, the data of the present study indicate that moderate, clinically stable COPD is associated with stable induced-sputum inflammatory markers. The quantification of most cellular and soluble components of induced sputum in stable COPD sampled on separate occasions over 5 weeks is sufficiently reproducible. In accordance with short-term observations, serial monitoring of inflammation in COPD using surrogate markers of induced sputum is a suitable tool for clinical routine, research purposes, and clinical trials.

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