Granulocyte markers in induced sputum in patients with respiratory disorders and healthy persons obtained by two sputum-processing methods

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Induced sputum is increasingly used to detect and monitor airway inflammation in respiratory diseases. However, the processing of sputum has been rather laborious for clinical practice.

The aim of this study was to improve the practicality of induced-sputum studies by simplifying sample processing. Eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), neutrophil lipocalin (HNL) and myeloperoxidase (MPO) were used as biochemical markers of airway inflammation and the results of the study method were compared with a previously validated (reference) method.

Induced sputum was obtained from 42 healthy controls, 10 subjects with acute respiratory infection, eight patients with chronic obstructive pulmonary disease (COPD) and 17 asthmatics. The sputum sample was divided into two parts and treated either: (i) by the reference method (released markers), where sputum was homogenized with dithiotreitol and centrifuged to yield cell-free supernatant and a cell pellet, or (ii) by the study method (total markers), where the cells were lysed after homogenization so that cell-associated markers were released and solubilized. For comparison, the four biochemical markers were measured in sputum supernatant and in sputum lysate. The differential cell count was performed from the cell pellet in the reference method. Repeatability was assessed in a group of 16 subjects. The effect of reagents and the recovery of assays were also evaluated.

Released and total markers correlated well (ECP $r_s=0.80$, $P<0.0001$; EPO $r_s=0.49$, $P<0.0001$; HNL $r_s=0.87$, $P<0.0001$; MPO $r_s=0.71$, $P<0.0001$). Incubation with dithiotreitol and lysis reagent had no negative influence on marker assays. The within-subject variability of total ECP, MPO and HNL in both methods was small in two measurements taken 1 week apart.

The study method, measuring total inflammatory markers, gave comparable results to the reference method, measuring released markers. In the study method the sputum processing was simplified, which may improve its applicability.

Key words: asthma; chronic obstructive pulmonary disease; eosinophil cationic protein; eosinophil peroxidase; induced sputum; infection; human neutrophil lipocalin; myeloperoxidase.

Introduction

Characteristic features of asthma and chronic obstructive pulmonary disease (COPD) are airflow limitation and bronchial inflammation. In these conditions increased numbers of activated inflammatory cells are found in bronchial biopsy specimens, in bronchoalveolar lavage (BAL) fluid, in sputum and in severe cases in peripheral blood (1–4). In asthma, eosinophils are considered to be the effector cells, whereas the role of neutrophils is more controversial. In patients with COPD, respiratory infections or asthma exacerbations increase the numbers of neutrophils that are present in the airways. Both eosinophils and neutrophils contain in their intra-cellular granules various mediators that are released on activation.

A practical method is needed to evaluate airway inflammation and to identify predominant granulocytes in different clinical situations. Bronchoalveolar lavage and bronchial biopsies have been widely used to obtain samples and specimens to study cellular and biochemically soluble granulocyte markers, but the invasiveness of bronchoscopic methods limit their usage. Inducing sputum is a less invasive and a safe method to investigate airway inflammation (4). It has been shown to be clinically valid and reliable (5–7) and it has given results comparable to those obtained
by bronchoscopic methods (8–11). Induced-sputum samples are more concentrated in cells and biochemical constituents than BAL samples obtained by fibre-optic bronchoscopy (9). Sputum is derived from the diseased organ and reflects local inflammation better than measurements from blood (12).

Sputum processing for biochemical and cellular analysis has varied (4,7,13–18), and information obtained from different studies cannot be accurately compared. Hargreave et al. have validated the processing method for cellular and biochemical measurements (7,19). The method was modified for sputum induction and processing to improve its clinical applicability by the authors.

The study was designed with two objectives: (i) to compare cellular profiles and biochemical markers for eosinophils (ECP, eosinophil cationic protein; EPO, eosinophil peroxidase) and neutrophils (MPO, myeloperoxidase; HNL, human neutrophil lipocalin) by two sputum-processing methods, and (ii) to validate the sputum processing of the study method. The patients included had acute respiratory infection, COPD or asthma. A group of healthy subjects was also included.

Methods

SUBJECTS AND CLINICAL METHODS

The study population consisted of 42 healthy subjects, 10 patients with acute respiratory infections, eight with COPD, and 17 with asthma (Table 1). All subjects gave informed consent for the study, which was approved by the hospital ethics committee.

Healthy subjects with no history of asthma, other respiratory symptoms or respiratory infection within 4 weeks were recruited from the hospital staff and their relatives. Their peak expiratory flow (PEF) values (Spira Peak Flow®, Hämeenlinna, Finland) were within the normal range (20). Ten healthy subjects with acute respiratory tract infection for less than 1 week were also recruited. The microbial aetiology of the infection was not assessed, but symptoms were characteristic of acute viral infection. These patients had normal PEF values. Eight patients with a previous diagnosis of COPD (21) and a smoking history of 20 to 80 pack years were included. They were clinically stable and their mean forced expiratory volume in 1 sec (FEV₁) was 43% of predicted volume with less than 10% bronchodilatation response to inhaled salbutamol (Buventol Easyhaler®, 200 microg/dose, Orion Pharma, Espoo, Finland). All asthma patients had reversible airway obstruction with either at least 15% bronchodilatation response in FEV₁ to 200 µg inhaled salbutamol or at least 20% diurnal variation in PEF values, for at least 3 days during a 2 week follow-up period. All asthmatics showed increased bronchial responsiveness to inhaled histamine with means PD20FEV₁ of 0.35 mg (22). None of the COPD and asthma patients had received anti-inflammatory medication (inhaled corticosteroid, sodium cromoglycate or sodium nedocromile) or had had an exacerbation or respiratory infection within 4 weeks.

SPUTUM INDUCTION

Sputum was induced by inhalation of 5 ml of 3% NaCl solution using a small ultrasonic nebulizer (Omron U1, Omron, Germany). According to the manufacturer, the output is about 0.25 ml min⁻¹ and the mean mass media of nebulized saline solution ranges from 1 to 7 μm. Peak expiratory flow values were measured before and after induction to ensure the procedure was safe. No pre-medication was used. Subjects were asked to cough during and after inhalation and to expectorate into empty containers.

SPUTUM PROCESSING

Sputum samples were transferred to a Petri dish and the more viscous parts were collected using forceps. Sputum plugs (selected from saliva) were first mixed with forceps and then divided into two parts to compare two different methods for sputum processing. Sample parts were processed either: (i) by the reference method described by Pizzichini et al. (7) to collect cells and cell-free supernatant (released markers), or (ii) by the study method, where the

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy individuals</th>
<th>Respiratory infection</th>
<th>COPD</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42.0 (29–61)</td>
<td>38.9 (19–56)</td>
<td>68.2 (58–76)</td>
<td>42.2 (18–72)</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>33/9</td>
<td>6/4</td>
<td>5/3</td>
<td>10/7</td>
</tr>
<tr>
<td>Smoker*</td>
<td>1 (2)</td>
<td>1 (10)</td>
<td>8 (100)</td>
<td>6 (35)</td>
</tr>
<tr>
<td>Atopic*</td>
<td>7 (17)</td>
<td>1 (10)</td>
<td>0 (0)</td>
<td>9 (53)</td>
</tr>
<tr>
<td>PEF (% of predicted)</td>
<td>103.6 (87–120)</td>
<td>101.8 (93–116)</td>
<td>51.3 (28–64)</td>
<td>83.7 (53–109)</td>
</tr>
<tr>
<td>FEV₁ (% of predicted)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>42.7 (27–66)</td>
<td>85.0 (69–101)</td>
</tr>
</tbody>
</table>

Data are expressed as mean and range or number and percentage of patients; COPD: chronic obstructive pulmonary disease; defined as at least one positive allergy skin prick test (wheal ≥3 mm); reference values from Viljanen (34); n.d. not done.
sample was treated first with dithiotreitol (DTT) as well as with a cetyl-N,N,N-trimethylammonium bromide (CTAB) detergent in order to lyse the cells and then to collect supernatant markers (total markers).

In the reference method (released markers), a four-fold volume of 6.5 mmol 1⁻¹ dithiotreitol (Sputolyisin®; CalbioChem, LaJolla, CA, U.S.A.) diluted 10-fold in distilled water, was added to the sputum sample and the mixture was incubated for 15 min in a roller mixer at room temperature. An equal volume of phosphate-buffered saline (PBS) was added, and incubation was continued for another 5 min. The mixture was filtered using 53 μm nylon-mesh filter (Nybolt PA-53/35, Seidengaze, Germany). Sputum cells were separated by centrifugation at 800 g for 10 min and the sputum supernatant was collected for measurements of fluid-phase markers. Cells obtained after centrifugation were resuspended in 1 ml of PBS and the total cell number was determined using a haemocytometer. Viability was measured by the Trypan Blue exclusion test. The cell suspension was cyt centrifuged (Cytospin 3, Shandon, Astmoor, U.K.) on to the Vectabond-treated (Vectabond® Reagent, Vector Laboratories, Inc., CA, U.S.A.) microscope slides at 450 rpm for 4 min. Slides were air-dried for at least 30 min. One slide was used for differential staining with the May-Grünwald-Giemsa (MGG) method. Two slides were fixed using Carnoy’s fixative and stained using toluidine blue for detection of metachromatic cells (basophils, mast cells). A minimum of 400 non-squamous cells was counted in MGG-stained slides and 1500 in toluidine blue-stained slides with a standard light microscope. Results were expressed as percentages of the total non-squamous count.

In the study method (total markers), a four-fold volume of 6·5 mmol 1⁻¹ DTT was added to the sputum sample, as in the reference method, and the mixture was incubated for 15 min in a roller mixer at room temperature. Differently from the reference method, a five-fold volume of CTAB reagent (0·5% CTAB, 0·4% HSA 100 mm PBS, pH 7·2) was added after 15 min and the mixture was incubated for 1 h in a roller mixer. By this measure, cells were lysed and granulocyte markers inside and outside the cells were released and solubilized (23). Sputum supernatant was separated from cell debris by centrifugation at 800 g for 10 min. The resulting clear suspension was aspirated, aliquoted and stored in Eppendorf tubes at −20°C for later assays.

MEASUREMENTS OF BIOCHEMICAL MARKERS

Concentrations of ECP (μg l⁻¹) in thawed supernatants were determined using an immunoassay kit (CAP System FEIA ECP, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), and levels of MPO (μg l⁻¹) using a double-antibody RIA kit (MPO RIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Concentrations of EPO (μg l⁻¹) and HNL (μg l⁻¹) were determined using prototype kits (CAP System FEIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), as previously described (24). Results were adjusted for dilutions and all measurements were performed in duplicate.

CONTROL EXPERIMENTS

To evaluate the within-subject variability of induced sputum, a group of 16 subjects gave induced-sputum samples twice in a 1-week interval. The group consisted of 12 healthy subjects (mean age 44 years, 11 females) with normal PEF values, and four asthma patients (mean age 42 years, three females) with the mean FEV₁ 75.5% predicted. Sputum samples were analysed with both the reference method and the study method. To examine the effect of DTT and CTAB on marker assays of total ECP, a standard curve with DTT and CTAB was made and compared with the curve with physiological saline. Recovery of ECP was examined by adding stock solutions of ECP standards to three different sputum samples pre-incubated with DTT and CTAB. Control experiments for EPO, MPO and HNL were performed analogously.

DATA ANALYSIS

Data are expressed as means or medians and ranges, as some values were below the lowest standard point. All results were included in data analysis. Results with immeasurable values were processed by giving the numerical value of the lowest standard point. Significances of differences between the four study groups were first analyzed using Kruskal-Wallis one-way analysis by ranks. When significant differences between groups were observed, inter-group comparisons were assessed by a non-parametric method using the Wilcoxon–Mann U-test. For correlation between variables the Spearman rank-order test was used. A two-tailed P-value of 0·05 or less was considered significant. Repeatability of marker measurements was expressed as intra-class correlation coefficient (R_i) (25) using log-transformed values.

Results

All subjects tolerated the sputum-induction procedure well. The median percentage of squamous epithelial cell contamination was 5.4% (range 0–72%) from all subjects (n = 77). In all subjects the cell viability was over 80%. Patients with asthma and COPD showed no significant differences in the mean proportions of sputum eosinophils, but both groups had more eosinophils than patients with respiratory infection or healthy persons (Table 2).

CORRELATION OF RELEASED BIOCHEMICAL MARKERS TO TOTAL MARKERS

Concentrations of both released and total ECP and EPO were highest among patients with COPD. Patients with respiratory infection and COPD had significantly higher
TABLE 3. Concentrations of inflammatory markers in induced sputum obtained by the reference method (measuring released markers) and the study method (measuring total markers)

<table>
<thead>
<tr>
<th>Inflammation markers</th>
<th>Healthy individuals</th>
<th>Respiratory infection</th>
<th>COPD</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=42</td>
<td>n=10</td>
<td>n=8</td>
<td>n=17</td>
</tr>
<tr>
<td>Total ECP*</td>
<td>572 (68–2706)</td>
<td>4937 (397–21160)</td>
<td>5840 (681–15400)</td>
<td>2485 (386–69940)</td>
</tr>
<tr>
<td>Released ECP*</td>
<td>140 (19–985)</td>
<td>1329 (67–14202)</td>
<td>2104 (88–3726)</td>
<td>748 (60–5647)</td>
</tr>
<tr>
<td>Total EPO*</td>
<td>45.0 (&lt;25.0–6830)</td>
<td>47.7 (37–162)</td>
<td>3550 (380–7435)</td>
<td>632 (45–41654)</td>
</tr>
<tr>
<td>Released EPO*</td>
<td>5.2 (&lt;4.5–121)</td>
<td>35.6 (&lt;4.4–1436)</td>
<td>166.5 (9–517)</td>
<td>83.5 (5.1–2927)</td>
</tr>
<tr>
<td>Total HNL***</td>
<td>6199 (1146–19324)</td>
<td>31789 (1956–138077)</td>
<td>18774 (1645–44898)</td>
<td>4059 (2446–20330)</td>
</tr>
<tr>
<td>Released EPO*</td>
<td>5549 (1005–27855)</td>
<td>26249 (1854–95580)</td>
<td>14304 (2260–34182)</td>
<td>6215 (2448–22643)</td>
</tr>
<tr>
<td>Total MPO*</td>
<td>178 (&lt;80–1524)</td>
<td>3402 (146–57600)</td>
<td>7125 (395–58000)</td>
<td>192 (&lt;80–21800)</td>
</tr>
<tr>
<td>Released MPO*</td>
<td>&lt;72 (&lt;72–1555)</td>
<td>599 (–72–4027)</td>
<td>1490 (144–3429)</td>
<td>&lt;72 (&lt;72–72)</td>
</tr>
<tr>
<td>Total ECP/MPO*</td>
<td>2.2 (0–3–11.2)</td>
<td>0.9 (0.2–6.1)</td>
<td>0.7 (0.2–11.5)</td>
<td>7.6 (0.2–219.6)</td>
</tr>
<tr>
<td>Total ECP/HNL*</td>
<td>0.09 (0.02–0.58)</td>
<td>0.15 (0.05–0.31)</td>
<td>0.34 (0.29–0.45)</td>
<td>0.48 (0.05–6.46)</td>
</tr>
</tbody>
</table>

Data are expressed as median and range. *P<0.001, **P<0.02 for differences between the four groups, Kruskal-Wallis; COPD: chronic obstructive pulmonary disease.

concentrations of both released and total MPO and HNL (Table 3).

Correlation between released and total marker concentrations [Fig. 1(a–d)] was excellent for ECP (r=0.80, P<0.0001) and for HNL (r=0.87, P<0.0001), good for MPO (r=0.71, P<0.0001) and moderate for EPO (r=0.49, P<0.0001), when all study groups were included in the evaluation. Not surprisingly, correlation varied between study groups and markers [see legends for Fig. 1(a–d)]. For example, released EPO concentrations correlated significantly with total EPO concentrations in the asthma group, but not so well in patients with respiratory infection or COPD or in healthy persons. On the other hand, strong correlation for released and total ECP and for released and total HNL results was observed in all groups.

The ratio of eosinophil and neutrophil markers, total ECP/MPO, was able to differentiate healthy persons from all patient groups. The ratio of total ECP/HNL differentiated healthy persons from patients with asthma or COPD. Both indices demonstrated significant differences between groups (total ECP/MPO: P<0.0001; total ECP/HNL: P<0.0001; Kruskal-Wallis).

CORRELATION OF RELEASED AND TOTAL BIOCHEMICAL MARKERS TO SPUTUM CELLS

Released and total ECP and EPO correlated significantly with eosinophil percentages (r=0.52 for released ECP, P<0.0001; r=0.62 for total ECP, P<0.0001; r=0.67 for released EPO, P<0.0001; r=0.69 for total EPO, P<0.0001) when all study groups were included. Similarly, released and total HNL and MPO correlated to neutrophil percentages (r=0.52 for released HNL, P<0.0001; r=0.65 for total HNL, P<0.0001; r=0.56 for released MPO, P<0.0001; r=0.56 for total MPO, P<0.0001). The power of correlation somewhat varied between the study
group and marker. For instance, in asthmatics, but not in patients with COPD and in healthy individuals, significant correlation between eosinophil percentage and total ECP and EPO concentrations was found.

CONTROL EXPERIMENTS AND REPEATABILITY

The addition of DTT and CTAB to the reference protein did not change the shape of standard curves (data not shown). Recoveries for total markers were acceptable (between 80% and 120%). For ECP it was 102.1% (mean), for EPO 96.6%, for HNL 100.5% and for MPO 99.7%, indicating that corresponding proteins were not bound to pre-treated sputum sample influencing the analysis.

The repeatability (Ri) was good for released ECP (0.85), total ECP (0.78), released MPO (0.97), total MPO (0.90), released HNL (0.91), total HNL (0.61), and poorer for released EPO (0.52) and total EPO (0.22).

Discussion

The previously validated sputum processing method (7) was modified in order to improve its clinical applicability. In this modified method, sputum specimens for measurement of various biochemical markers can be frozen and stored after collection, so that specimens can be taken in one place (e.g. in a healthcare centre) and processed and analysed in another (e.g. in a central laboratory). The study method for sputum processing gives results comparable to those obtained by the reference method (7). By measuring one eosinophil-activation marker, ECP, and one neutrophil marker, MPO, with a commercially available kit, airway inflammation can be studied with reasonable accuracy. Incubation of the reference protein with DTT and CTAB showed no negative influence on the marker assays. Recovery of four markers (ECP, EPO, MPO and HNL) ranged from 96.6% to 102.1%.

Sputum processing for obtaining cytopsin slides and differential cell counts is laborious and can be used in larger laboratories. The prerequisite for reliable cytopsin slides is processing the sputum immediately rather than later. This is only possible in large laboratories. The advantage of the study method (which measures total markers) over the reference method (which measures released markers) is its relative simplicity allowing small laboratories, that do not have cytocentrifuge to use it. The amount of inflammatory cells can be studied in a semi-quantitative manner from smears made prior to freezing using eosin-methylene-blue staining (26). Sputum induction and collection of sputum plugs is the same in both methods.
Recently, considerable attention has been paid to the methods of sputum processing. The first methodological choice, affecting both cellular and biochemical marker results, is to use the entire sample, including variable amounts of saliva (13), or to select a part (plugs) of the sample (7). Concentrations for biochemical markers have been assayed from (i) sputum supernatants (7,13), from (ii) the entire expectorates including cells and supernatant (16,17), and from (iii) cell pellets (27). The first alternative measures the extracellular, secreted form of a marker, which may reflect the degree of cell activation. However, it may be difficult to assess whether markers have pre-existed in the airways or were released because of the hypertonic saline inhalation or sample treatment, as has been hypothesized for albumin and fibrinogen (28). The second alternative measures both intra- and extracellular marker concentrations and thus reflects both the activity and the size of the source-cell population in the airways. The third alternative is suggested to correlate with absolute number of sputum cells.

The authors’ approach was to measure the total marker concentrations (intra- and extracellular) in sputum selected from saliva. It was found that analysing the whole sample content of plugs is simple and gives results comparable to markers released in the supernatant. All sputum-processing methods with differential cell counting published so far are relatively laborious. The relative simplicity of our method may be an advantage if the method is recommended for wider use.

Sputum plugs were first treated with DTT and then subjected to CTAB. The DTT treatment is necessary since liquidification of sputum plugs is desired prior to CTAB treatment. The detergent was added in order to lyse the cells and to liberate biochemical markers possibly engulfed by other cells. CTAB was chosen as a detergent since sticky proteins such as ECP can be efficiently recovered from the cell pellet by 1-h CTAB treatment, as shown by Carlson et al. (23). In-vitro studies have shown that at least apoptotic eosinophils and neutrophils are recognized and phagocytosed by macrophages (29,30) and they could therefore be an additional source of granule proteins when using this extraction procedure. The highly cationic proteins EPO and ECP have a tendency to stick to cell surfaces and to form insoluble aggregates. Tendency to bind to surfaces of test tubes has also been noticed during the purification of EPO (31). Control experiments showed that neither DTT nor CTAB had any negative effects on marker assays.

Correlation between released and total marker concentrations was excellent, but varied depending both on the study group and the cellular source of a marker in question. In asthma and COPD, the two eosinophil-derived markers, released and total ECP, as well as released and total EPO, correlated significantly. Strong correlation between released and total MPO, as well as released and total HNL, was observed in patients with infection and with COPD.

An index that evaluates the amount of eosinophils in relation to neutrophils may be useful in differential diagnostics. A ratio of total ECP to total HNL revealed a less clear difference between the study groups.

The findings, with regard to the cellular and biochemical composition of induced sputum in patients with asthma and COPD, and in healthy controls, are in line with reports by others (12,13,18). In the present study, sputum eosinophils correlated significantly with total ECP and total EPO in patients with asthma. Concentrations of HNL and MPO were significantly correlated with neutrophil numbers in COPD patients and in subjects with respiratory infection. This suggests that eosinophil inflammation associated with asthma and neutrophil inflammation associated with airway infection and with COPD can be detected by sampling the airways using the two different techniques presented here.

In a study by Lacoste (2), neither the numbers of neutrophils nor the concentrations of MPO were increased in BAL fluids or in mucosal biopsies in most asthmatic patients. In contrast, patients with COPD showed increased numbers of neutrophils and concentrations of MPO in BAL fluids (2). In the present study, released-MPO levels were undetectable in all asthma patients and in 66% of control subjects, but never in COPD patients or subjects with respiratory infection. The strong correlation observed between MPO and ECP in patients with acute respiratory infection suggests that factors that prime neutrophils during respiratory infection may also have an effect on eosinophils. Venge et al. have recently reported an active uptake of ECP by neutrophils (32). This might partly explain the observed correlation, since ECP is suggested to co-localize with MPO in the secondary granules of neutrophils. The absence of increased levels of EPO at the same time could be explained by the unresponsiveness of the mechanisms leading to EPO release, as recently suggested (33).

Repeatability of differential cell counts and measurements of soluble inflammatory markers have been good in samples obtained on different days from clinically stable patients (5,7). A slightly better repeatability was reported in moderate-to-severe asthmatics compared with those with mild asthma (5). A good repeatability for all marker measurements was found except for EPO, irrespective of the technique of sputum processing used.

In conclusion, a simplified method for measuring granulocyte markers has been described. Total marker assays were able to differentiate patients with respiratory disorders from healthy individuals. Total marker assays also gave comparable results with previously established released-marker assays. The results suggest that the induced sputum method with total marker assays may be used in clinical practice.

Acknowledgements

We thank Professor Per Venge for HNL assays and for his valuable advice and comments of this manuscript. Dr Katri Heikkinen and Dr Pekka Saarelainen are acknowledged for recruiting patients. The Research Institute of Helsinki
University Central Hospital, the Finnish Allergy Research Foundation and the Finnish Society of Allergology and Immunology funded the research.

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