Induced sputum, bronchoalveolar lavage and blood from mild asthmatics: inflammatory cells, lymphocyte subsets and soluble markers compared

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ABSTRACT: Airway inflammation in asthma can be measured directly by invasive bronchoalveolar lavage (BAL), directly and relatively noninvasively by induced sputum and indirectly from peripheral blood. We compared cellular and fluid phase indices of inflammation in induced sputum, BAL and blood from 11 adults with mild stable asthma.

On one day, induced sputum selected from saliva was collected and on the next, blood and BAL. Median results of sputum compared with BAL showed a higher number of nonsquamous cells (5.3 versus 0.8x10^6 cells·mL⁻¹, p=0.003), more neutrophils (34.3 versus 1.0%, p<0.001), CD4+ and CD19+ T-cells (76.5 versus 54.7%, p=0.01 and 5.2 versus 1.1%, p=0.03, respectively), fewer macrophages (60.3 versus 95.0%, p=0.002) and markedly higher levels of eosinophil cationic protein (ECP) (264 versus 2.0 µg·L⁻¹, p<0.001), tryptase (17.6 versus 2.2 UI·L⁻¹, p<0.001) and fibrinogen (1,400 versus 150 µg·L⁻¹, p=0.001). Sputum and BAL neutrophils and CD4+ T-cells were strongly correlated. Sputum and BAL differed from blood by having higher proportions of T-cells (94.9 and 98.9% versus 87.7%, p=0.002) and lower proportions of CD19+ T-lymphocytes (p=0.04 and 0.006). Sputum also differed from blood by having higher proportions of CD4+ T-cells (76.5 versus 51.4%, p=0.001), lower proportions of CD8+ cells (24.0 versus 40.3%, p=0.04) and a higher CD4+/CD8+ ratio (3.3 versus 1.4, p=0.01).

We conclude that in mild asthmatics, sputum, bronchoalveolar lavage and blood measure different compartments of inflammation. Induced selected sputum has the advantage over bronchoalveolar lavage of higher density of cell recovery and stronger signal for fluid-phase markers.


Airway inflammation is an important determinant of asthma severity and eventual airway remodelling. Many recent studies using bronchoscopy to obtain bronchial biopsies and bronchoalveolar lavage (BAL), have demonstrated the inflammatory features of milder asthma including the accumulation of eosinophils, mast cells and lymphocytes and their activation markers [1, 2]. The recent recognition that sputum can be processed to give reliable cell and fluid-phase measurements of inflammation [3, 4] has resulted in the need to make comparisons with measurements in BAL, bronchial biopsies and blood. Four studies have compared induced sputum with BAL or bronchial biopsies with respect to cell counts [5–8], one of these has compared fluid-phase eosinophil cationic protein (ECP), albumin and mucin-like glycoprotein [5] and one has compared lymphocyte subsets and activation markers [8]. The results indicate that the specimens measured different compartments in the airways and that the magnitude of signal in one compartment does not necessarily correlate with that of another.

The present study was begun before the above reports were published. We compared inflammatory indices in induced sputum with those in BAL and blood in 11 mild clinically controlled asthmatics. The study differed from those mentioned above by selecting sputum from the expectorator to minimize the confounding influence of saliva on fluid-phase measurements, by measuring fluid-phase tryptase and fibrinogen and by comparing sputum and BAL lymphocyte subsets with those in peripheral blood.

Methods

Subjects

Eleven nonsmoking adults with stable asthma were recruited from patients and staff of the Firestone Regional Chest and Allergy Unit (table 1). All subjects had a diagnosis of asthma established symptomatically by episodic
wheeze, chest tightness and/or dyspnoea and objectively confirmed by methacholine airway hyperresponsiveness (provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second; % pred: percentage of predicted value; PC20: provocative concentration of methacholine causing a 20% fall in FEV1; Ex: exsmoker (>6 months); Non: nonsmoker; BUD: budesonide (µg·day⁻¹); β₂, p.r.n.: inhaled β₂ agonist, as needed; BDP: beclomethasone dipropionate (µg·day⁻¹).

**Sputum induction**

Sputum was induced by the inhalation of hypertonic saline as described by Ps et al. [13]. Subjects were premedicated with inhaled salbutamol (200 µg). The aerosol was generated by a Fison® ultrasonic nebulizer (Canadian Medical Products Ltd., Markham, Ontario, Canada) with an output of 0.87 mL·min⁻¹ and a mass median aerodynamic diameter of 5.58 µm. The method was slightly modified by inhaling increasing concentrations of saline (3, 4, and 5%) for 7 min each through a mouthpiece without a valve or nose clips. After each inhalation period, an FEV1 value was measured for safety and the subjects were asked to blow their nose and rinse their mouth with water and swallow it to reduce contamination of the sputum specimens with post-nasal drip and saliva, and to try to cough sputum into a container.

**Bronchoscopy**

Fibreoptic bronchoscopy was performed using an Olympus BF-T20D bronchoscope (Tokyo, Japan) to obtain BAL as previously described [14]. Briefly, fasting subjects withholding anti-asthma medications for 24 h had spirometry before and after salbutamol (400 µg) inhaled from a pressurized inhaler. They received premedication with atropine (0.6 mg i.m.) and topical anaesthesia of the upper airways was achieved with lidocaine gargle (2 mL of 4% lidocaine solution for 60 s) and four sprays of 1% lidocaine (10 mg·spray⁻¹). The nasal passages were lubricated with 2% lidocaine gel. Then the bronchoscope was passed through the nose to just above the glottis and 2 mL of 4% lidocaine solution was applied through the bronchoscope.
The bronchoscope was then wedged in the right middle lobe or lingula and five aliquots of 20 mL of sterile normal saline at room temperature were instilled and aspirated with gentle hand suction. Pooled samples were kept on ice and processed within 30 min.

**Sputum examination for cell content**

Sputum was selected from saliva [15] and processed within 2 h as described by Pizzichini et al. [3]. Briefly, sputum was treated with a volume (mL) of 0.1% dithiothreitol (DTT) (Sputalysin 10%, Calbiochem Corp., San Diego, CA, USA) equal to four times the weight (mg) followed by the addition of similar four volumes of Dulbecco’s phosphate buffered saline (D-PBS). The suspension was filtered through a 48 µm nylon mesh (B&SH Thompson, Scarborough, Ontario, Canada) and the resulting suspension was used for total cell count (TCC) and cell viability. The cell suspension was adjusted to 1.0 × 10^6 cells·mL^-1, and cytospins were prepared in a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA). The cytospins were stained with either Wright’s or toluidine blue for differential and metachromatic cell counts. Cell counts were performed on 400 and 1,500 nonsquamous cells, respectively. The remaining cell suspension was centrifuged at 3,000 revolutions per minute (rpm) for 4 min. The supernatant was aspirated and stored in Eppendorf tubes at -70°C for later assays. The cell pellet was resuspended in D-PBS and used for flow cytometry.

**BAL examination for cell content**

The volume of BAL fluid was recorded and centrifuged at -4°C at 1,000 rpm for 10 min. The supernatant was removed and stored at -70°C for fluid-phase assays. The cell pellet was washed with D-PBS, centrifuged for 10 min at 1,000 rpm and the supernatant discarded. The cell pellet was resuspended in 10 mL of D-PBS. Total cell counts, viability, differential cell counts and metachromatic cell counts were performed as described for sputum. The remaining suspension was used for flow cytometry.

**Flow cytometry in blood, sputum and BAL**

Flow cytometry was performed using a two-colour FACScan Analyzer (Becton Dickinson, Mississauga, Ontario, Canada). The instrument was calibrated using CaliBRITE beads and AutoCOMP software (Becton Dickinson, Mississauga, Ontario, Canada) for setting the photomultiplier tube voltages in order to obtain the correct mean fluorescence intensity, set the fluorescence compensation and check the instrument sensitivity. Blood lymphocytes were phenotyped by standard dual-colour flow cytometry procedures. Sputum and BAL cells were incubated in the dark at room temperature with 10 µL of monoclonal antibody for 15 min. The monoclonal antibodies used were conjugated to fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The panel consisted of: CD3/CD19, CD3/CD4, CD3/CD8, and immunoglobulin (IgG1/IgG2a (Becton Dickinson). Postincubation cells were washed with phosphate buffered saline (PBS) and fixed with 0.5 mL of 0.9% paraformaldehyde. Acquisition of data was with a single argon ion laser, 488 nm FACScan Analyzer (Becton Dickinson). Lymphocytes were distinguished on the basis of cell size (forward scatter) and cell granularity (side scatter). In sputum, to enhance the number of lymphocytes for analysis an acquisition gate was set using the lower third of the side scatter field. Ten thousand cells were acquired in list mode and further analysed using the CONSORT 30 software (Becton Dickinson) and a Hewlett-Packard (HP 9000 series 302) computer (Fort Collins, USA). In sputum and BAL, because of contamination with debris, CD3/CD19 lymphocytes could not be expressed as a percentage of the gate, but were recorded as a percentage of the cells detected by fluorescence [16]. We therefore expressed blood CD3/CD19 lymphocytes in the same way for comparison. Analysis was based on a minimum of 2,000, 400 and 3,000 lymphocytes in blood, sputum and BAL, respectively.

**Soluble markers in sputum and BAL**

The concentrations of ECP and tryptase in the thawed supernatant were determined using a sensitive radioimmunoassay (RIA, Kabi Pharmacia Diagnostics AB, Uppsala, Sweden). Fibrinogen was measured by a “sandwich” enzyme-linked immunosorbent assay (ELISA) assay using a rabbit anti-human fibrinogen antibody (Dako A080 (Dako Diagnostics, Mississauga, Ontario, Canada)). The limit of detection for the fluid-phase assays were 2.0 µg·L^-1, 0.79 ng·mL^-1 and 2 IU·mL^-1 for ECP, fibrinogen and tryptase, respectively. The results of sputum measurements were adjusted for the dilution factor of the procedure [3].

**Data analysis**

Results are reported as median and interquartile range (IQR) calculated using weighted averages. Significance was accepted at the level of 95%. Dependent variables with non-normal distribution were log transformed before analysis. Differences between induced sputum and BAL cells and fluid-phase markers were examined by paired t-test. Differences between induced sputum, BAL and peripheral blood lymphocyte subsets were tested by repeated measures analysis of variance (ANOVA); the source of significant variation was identified by the Student-Newman Keuls procedure to adjust the significance for multiple comparisons [17]. The agreement between measurements was calculated by intraclass correlation co-efficient (R) [18] and graphically displayed as suggested by Bland and Altman [19]. The correlations were examined by Spearman rank correlation coefficient (r_s). Only correlations with an r_s value above 0.50 and a p-value <0.01 were considered significant.

**Results**

Measurements in sputum, BAL and peripheral blood were performed successfully in all but one patient who did not have a suitable sputum sample for flow cytometry. Both sputum induction and BAL procedures were well tolerated by all subjects.
Table 2. – Induced sputum, bronchoalveolar lavage and blood inflammatory indices

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<th>Neu</th>
<th>Mac</th>
<th>Try</th>
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<th>TCC</th>
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Cell counts (x106 cells·mL⁻¹), Eos: eosinophils; Neu: neutrophils; Mac: macrophages; Try: tryptase; ECP: eosinophil cationic protein; IQR: interquartile range; ECP measured in haemolized serum.

Sputum versus BAL cells

Cells in sputum and BAL had good and similar viability (78.5 (17) versus 78.2 (11.9)%, p=0.9). In comparison with BAL, sputum had more cells per millilitre (p=0.003) and a higher proportion of neutrophils (p<0.001), CD4+ and CD19+ T-cells (p=0.01 and p=0.03, respectively). Compared with BAL, sputum had higher CD4+/CD8+ T-lymphocyte ratios (3.3 (4.3) versus 1.6 (1.6), p=0.02) (tables 2 and 3, fig. 1). The proportion of macrophages was lower (p=0.002 and p=0.04, respectively) (fig. 1) and there was no statistically significant difference in the proportion of eosinophils (p=0.1) or lymphocytes (p=0.9) (table 2, fig. 1), metachromatic cells (1.0 (0.2) versus 0 (0.5)%, p=0.1) and bronchoepithelial cells (0 (0.5) versus 0 (0.5)%, p=0.6). Significant positive correlations were observed between sputum and BAL neutrophils and sputum and BAL CD4+ lymphocytes only (fig. 2). With the exception of cell viability (r=0.6) and CD3+ lymphocytes (r=0.75), the agreement of these cells in sputum and BAL was poor (R<0.5 for all) (fig. 1).

Sputum versus BAL fluid-phase markers

Sputum fluid-phase levels of ECP (p<0.001), tryptase (p<0.001) and fibrinogen (p=0.001) were higher than in BAL (table 2, fig. 1). Sputum ECP correlated with the proportion of eosinophils (rs=0.78, p=0.004) and fibrinogen (p=0.001) were higher than in sputum or BAL lymphocyte subsets measurements on the other. Compared with BAL, sputum had more CD4+/CD8+ T-lymphocytes only (fig. 2). With the exception of cell viability (r=0.6) and CD3+ lymphocytes (r=0.75), the agreement of these markers in sputum and BAL was also poor (fig. 1).

Sputum and/or BAL versus blood lymphocyte subsets

CD3+ T-lymphocytes were higher in sputum and BAL than in peripheral blood (p=0.002 for both comparisons) (table 3). CD4+ T-lymphocytes (p=0.01, table 3) and CD4+/CD8+ ratio (3.3 (4.3) versus 1.5 (1.6), p=0.01) were higher in sputum than blood while CD8+ cells were lower (p=0.002 and p=0.04, respectively) (fig. 1) and the proportion of these cells correlated between the two specimens, the levels of fluid-phase components did not. The proportion of eosinophils correlated with the levels of fluid-phase ECP in sputum but not in BAL. Both sputum and BAL had more T (CD3+) lymphocytes than peripheral blood and sputum had more CD4+ T-cells and B (CD19+) cells. These data support and add to those in previous reports [5–8]. They indicate that the results from different specimens measure different compartments and,

Discussion

The results demonstrate that induced sputum contains a greater proportion of neutrophils and CD4+ T-cells and higher concentrations of ECP, tryptase and fibrinogen than BAL in subjects with mild controlled asthma. While the proportion of these cells correlated between the two specimens, the levels of fluid-phase components did not. The proportion of eosinophils correlated with the levels of fluid-phase ECP in sputum but not in BAL. Both sputum and BAL had more T (CD3+) lymphocytes than peripheral blood and sputum had more CD4+ T-cells and B (CD19+) cells. These data support and add to those in previous reports [5–8]. They indicate that the results from different specimens measure different compartments and,
Fig. 1. – The differences (log values except for neutrophils and macrophages) between induced sputum (IS) and bronchoalveolar lavage (BAL) total cell count (TCC), eosinophils, neutrophils, macrophages, eosinophil cationic protein (ECP), and fibrinogen are plotted as a function of the mean of two values. Intraclass correlation coefficients (R) are shown. ——: mean difference; - - - : ±2SD of mean difference.

Table 3. – Induced sputum, bronchoalveolar lavage (BAL) and blood inflammatory indices

<table>
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<th>Subject No.</th>
<th>Sputum %</th>
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Median: 94.9, IQR: 5.2, 76.5, 24.0, 98.9, 1.1, 59.7, 36.5, 87.7, 8.1, 51.4, 40.3

NA: not available; IQR: interquartile range; #: p<0.05 between sputum and blood; §: p<0.05 between BAL and blood.

Thus, may not necessarily correlate. In addition, the observed correlations, or lack thereof, may be due to subject selection, the measurement property of the specimen and the confounding influence of diluting fluids with sputum and BAL specimens.

This study was designed to minimize confounding factors. The sputum was induced 24 h before BAL to minimize day-to-day variability. It seems unlikely that this procedure would alter the BAL counts. This is supported by the observations of FAHY et al. [5] who compared BAL markers after sputum induction in five asthmatic subjects using a volume of hypertonic saline three times the volume we used, without differences compared with a previous baseline BAL. We have carried out examination of sputum selected from the remainder of the expectorate to minimize dilution with saliva, using a method which is very efficient in doing this [15]. We could not, however, avoid the influence of the variable dilution of the lavage fluid on the BAL fluid-phase levels.

The subjects selected for the study had asthma that was controlled and stable. As a result, the proportion of eosinophils in sputum was normal in seven of 11 subjects. This may explain why the proportion of eosinophils was not higher in sputum than in BAL. The higher proportion of neutrophils in sputum was controlled and stable. As a result, the proportion of eosinophils was not higher in sputum than in BAL. The higher proportion of eosinophils in sputum was normal in seven of 11 subjects.

[5] who compared BAL...
and BAL suggest that sputum samples the larger airways while BAL particularly samples the peripheral airways. Why there was not a greater proportion of lymphocytes in BAL in the present study is not clear; one wonders whether it was a result of the smaller volume of lavage (100 mL) than used in other reports.

The measurement properties of the method of sputum examination we used, particularly reliability [3], are excellent. The repeatability of sputum specific cell counts [3] and lymphocyte subsets [16] is high, while the repeatability of cells and soluble markers in BAL, with the exception of eosinophils, is poor [21, 22]. Highly repeatable instruments are those with a high signal to noise ratio [23] which is accomplished with the method of sputum examination we used. In contrast, BAL has a low signal to noise ratio, which results from variability in lavage and recovered fluid [24–26]. This may explain why there was a good correlation between the low numbers of eosinophils and ECP levels in sputum but not in lavage.

In the present study we have demonstrated increased proportions of T-lymphocytes in induced sputum and BAL, and significantly higher proportions of CD4+ and CD19+ T-cells in sputum than BAL and peripheral blood. In agreement with a previous report by Grootendorst et al. [8] the proportion of CD4+ cells in induced sputum correlated with those in BAL. The same correlations were not observed with peripheral blood. This is not surprising since peripheral blood is less likely to reflect the inflammatory process in the airways [27, 28]. The higher concentration of T-cells in the airways is in keeping with the role of lymphocytes in the pathogenesis of asthma and it is in agreement with other studies examining lymphocyte subsets by flow cytometry in BAL [29] or induced sputum [30]. The lower proportions of B-cells in sputum in comparison with our previous report [16] may be a result of subject selection.

In conclusion, inflammatory indices in induced sputum selected from the expectorate differ from those in bronchoalveolar lavage and blood and do not necessarily correlate. These partially confirm the findings of other studies comparing bronchoalveolar lavage with induced expectorate. The differences between studies may be a
result of differences in subject characteristics and differences in the sputum and bronchoalveolar lavage samples examined. The lack of correlations between specimens may reflect different properties of each instrument or the type of airway sampling. If the first is present, these differences indicate that the instrument with better properties is superior because it enables more reliable measurements. If the later is true, and both instruments have similar properties, the measurements in each of the specimens give complementary information. These issues can be further evaluated by comparing the ability of each method to measure longitudinal changes in airway inflammation after an intervention.

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References