Comparison of Three Methods for Differential Cell Count in Induced Sputum*

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**Background:** Induced sputum (IS) using the cytospin technique has been extensively employed to characterize inflammatory airway diseases; however, procedures of cell enrichment based on cytospin increase the analytical costs and require slide processing within a short period of time after sampling.

**Study objectives:** To compare three different techniques for cytologic analysis of IS, and to determine the time required by each method and the costs involved.

**Design:** Cross-sectional study.

**Setting:** Tertiary-care university hospital.

**Patients and measurements:** Eighty-nine patients with asthma and 11 subjects without asthma were submitted to increasing hypertonic saline solution concentrations of 2, 3, 4, and 5% for 7 min for sputum induction. Samples were smeared without treatment with 0.1% dithiothreitol (DTT) [technique A], after treatment with DTT (technique B), and after treatment with DTT and cytospin (technique C). All slides were air-dried and stained with Leishman stain. Two independent observers counted at least 200 inflammatory cells on each slide.

**Results:** Eighty percent of the slides processed by techniques A and B and 65% of the slides processed by technique C represented sputum samples of acceptable quality. The eosinophil percentages in sputum obtained by techniques A and C were closely correlated, as also were those obtained by techniques B and C (r = 0.64 and r = 0.63, respectively; p < 0.01). There was a positive correlation for eosinophils when we compared techniques A and B (r = 0.57, p < 0.01). The neutrophil correlation was significant when the three techniques were compared (technique A vs technique B, r = 0.66; technique A vs technique C, r = 0.51; and technique B vs technique C, r = 0.57; p < 0.01). Bland-Altman analysis showed a good agreement for eosinophil and neutrophil counts when techniques A and B were compared to technique C.

**Conclusions:** The three techniques are good indicators of lung inflammation. Techniques A and B are less time consuming and are of lower cost. (CHEST 2003; 124:1060–1066)

**Key words:** asthma; eosinophils; induced sputum; neutrophils

**Abbreviations:** DTT = dithiothreitol; IS = induced sputum; PEFR = peak expiratory flow rate

Airway inflammation is considered to be a major factor in the pathogenesis of asthma. At present, airway inflammation assessment in asthma involves either invasive techniques such as fiberoptic bronchoscopy with BAL and/or bronchial biopsies, or less invasive techniques such as induced sputum (IS). Because of its low invasiveness, IS has been frequently used to study airway inflammation in asthma. Popov et al proposed a method of sputum examination involving the use of cytospin.
and found advantages over the use of simple smears. Several studies with IS or spontaneous sputum using the cytospin method of cell count have provided evidence of reproducibility\textsuperscript{11–13} and responsiveness\textsuperscript{14–18} and have been carefully validated.\textsuperscript{19,20} However, the study of bronchial smears using procedures of cell enrichment based on cytospin increases the analytical costs and requires slide processing within a short period of time after sampling. Thus, the cost/benefit relationship of cytospin procedures over the conventional smear preparation must be objectively evaluated. In this study, we addressed this question by comparing the results and costs—material and time of preparation—of three different techniques of slide preparation employed for cellular analysis of IS collected from healthy and asthmatic subjects.

**Materials and Methods**

**Subjects**

The study population consisted of 89 patients with asthma (mild, n = 32; moderate, n = 28; severe, n = 29) and 11 subjects without asthma randomly selected from those receiving medical assistance at the asthma outpatient clinic of the University Hospital (Hospital das Clínicas) of the School of Medicine of the University of São Paulo, from 1998 to 2001. All patients with asthma had diagnoses classified according to the 1997 clinical practice guidelines of the National Institutes of Health.\textsuperscript{21} Sputometry was performed on all subjects according to the American Thoracic Society\textsuperscript{22} standardization using a Koko spirometer (POS Instrumentation; Louisville, CO). Peak expiratory flow rate (PEFR) was monitored during sputum induction with a peak flowmeter (Mini-Wright; Clement Clark International; Harlow, Essex, England). The asthmatic patients were asked to provide a sample of IS and to complete a medical questionnaire. The patients were instructed to rinse their mouths with water before sputum induction to avoid as much as possible salivary contamination of sputum samples.\textsuperscript{8} All subjects were able to produce sputum after induction.

**Sputum Induction**

A spirometric measurement was performed; subsequently, all subjects inhaled two puffs of salbutamol, 200 μg each puff.\textsuperscript{23} Fifteen minutes later, a second spirometric measurement was made and the sputum induction procedure was started. We tried to collect as much sputum as possible after each inhalation, monitoring the peak flow every 7 min. The sputum was collected into a sterile container.\textsuperscript{24,25} The procedure was interrupted if a ≥20% fall in PEFR occurred or if there was clinical discomfort that did not respond to another puff of salbutamol, 200 μg. The sputum sample was considered adequate if it met the following criteria: sputum induction tolerated for at least 14 min, sputum volume >2 mL, presence of squamous cell ≤80%, at least 200 inflammatory cells per slide, and examination of sputum performed within 2 h.\textsuperscript{13}

**Sputum Processing**

Sputum samples were visually separated from saliva with the help of an inverted microscope\textsuperscript{26,27} and were divided into three aliquots to be evaluated by the three different techniques.

**Technique A**

The sample was spread over the glass slides prior to fixation and staining. The slide was air-dried, fixed, and stained with Leishman stain (Sigma; St. Louis, MO).\textsuperscript{27}

**Technique B**

A phosphate-buffered saline solution was prepared with dithiothreitol (DTT) (Sigma-Aldrich, Brazil) at 0.1% concentration and

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**Table 1—Characteristics of Subjects**

<table>
<thead>
<tr>
<th>Patient Groups</th>
<th>Sex, No.</th>
<th>Age Range (mean ± SD, yr)</th>
<th>FEV\textsubscript{1}, % Predicted</th>
<th>FEF\textsubscript{25–75%,} % Predicted</th>
<th>FEV\textsubscript{1}/FVC, % Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Female 7</td>
<td>19–34 (27.1 ± 5.5)</td>
<td>104.8 ± 16.7</td>
<td>95.2 ± 24.7</td>
<td>98.8 ± 9.2</td>
</tr>
<tr>
<td></td>
<td>Male 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild asthma</td>
<td>Female 23</td>
<td>13–47 (26.2 ± 8.3)</td>
<td>95.1 ± 20.9</td>
<td>64.5 ± 30.8</td>
<td>82.9 ± 20.6</td>
</tr>
<tr>
<td></td>
<td>Male 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate asthma</td>
<td>Female 20</td>
<td>15–70 (30.5 ± 16.9)</td>
<td>76.8 ± 19.2</td>
<td>46.6 ± 25.6</td>
<td>78.8 ± 15.4</td>
</tr>
<tr>
<td></td>
<td>Male 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe asthma</td>
<td>Female 24</td>
<td>12–60 (36.2 ± 12.0)</td>
<td>50.5 ± 20.4</td>
<td>24.8 ± 18.1</td>
<td>67.2 ± 15.0</td>
</tr>
<tr>
<td></td>
<td>Male 5</td>
<td></td>
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*Data are presented as mean ± SD unless otherwise indicated. FEF\textsubscript{25–75%} = forced expiratory flow between 25% and 75% of vital capacity.*

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added to an equal volume of sputum, and the mixture was briefly stirred with a vortex mixer. DTT is a sulfhydryl reagent that produces mucolysis by breaking disulfide bonds that crosslink glycoprotein fibers. Drops of this mixture were then spread over the glass slides, and the slides were air-dried, fixed, and stained with Leishman stain.

**Technique C**

The sample was treated with 0.1% DTT phosphate-buffered solution up to a ratio of 1:4 by volume. The mixture was then vortexed, and rocked for 20 min at 37°C. The clear cell suspension was filtered through a 48-μm nylon gauze (BBBH Thompson; Scarborough, ON, Canada) to remove debris and mucous. In this technique, samples were processed as soon as possible within 2 h. Total cell count was performed with a hemocytometer (Neubauer chamber). Percentage of viable cells were evaluated by trypan blue exclusion, and blue-stained cells were considered dead. The cell suspension was adjusted to 1.0 × 10⁶/mL. Approximately 100 μL of the cell suspension was placed in each cyto centrifuge cup. The slides were air-dried, fixed, and stained as described for techniques A and B.

**Cell Counts**

Cell counts were performed with a light microscope at 1,000 × magnification. At least 200 cells were counted by two investigators blinded to patient classification. Cells were classified as eosinophils, lymphocytes, neutrophils, macrophages, squamous cells, goblet, and ciliated cells based on their morphology.

**Statistical Analysis**

Data are presented as means and SDs. Cytologic data were analyzed statistically by Kruskal-Wallis nonparametric analysis of variance. To isolate the group or groups that differed from the others, a multiple comparison procedure (Dunn’s method) was used; p < 0.05 was considered statistically significant. The correlation among the three techniques studied was examined by Spearman rank correlation coefficients. Only correlations with r > 0.50 and p < 0.01 were considered significant. The agreement between the cytologic data obtained by techniques A and B and the established technique C was studied by Bland-Altman analysis. This method of analysis estimates the average bias of one method relative to the other and also how the methods are likely to agree for an individual. The time spent to obtain the results for each technique was compared by one-way analysis of variance followed by the Tukey test; p < 0.05 was considered statistically significant.

**RESULTS**

Only one patient presented a decline in PEFR > 20% during sputum induction (30%), and PEFR returned to baseline values 15 min after salbutamol administration. This patient had severe asthma and was not included in the analysis.

Eighty percent of the slides processed by techniques A and B and 65% of the slides processed by technique C had acceptable indexes of adequate sputum samples. These results pertain only to the slides that fulfilled the criteria of acceptability.

Mean total sputum cell viability by trypan blue exclusion was 50.00 ± 14.47%, 55.62 ± 20.70%, 50.33 ± 22.79%, and 53.18 ± 25.74% for control subjects and patients with mild, moderate, and severe asthma, respectively (p = not significant). Total cell counts were 1.87 ± 0.97, 3.37 ± 3.19, 4.55 ± 4.24, and 5.06 ± 4.07 × 10⁶/mL for control subjects and patients with mild, moderate, and severe asthma, respectively (p = not significant).

Figure 1 shows representative slides and photomicrographs of smears obtained by each of the three different techniques of analysis of induced sputum studied. Figure 2 shows the percentages of eosinophils, neutrophils, lymphocytes and macrophages in the induced sputum processed by the three techniques. The percentages of eosinophils were higher in patients with moderate and severe asthma compared to nonasthmatic subjects, and these differences were observed with the three techniques studied. The percentages of neutrophils, lymphocytes, and macrophages were similar when the four groups of subjects were compared. In fact, the only significant differences observed were in the percentage of neutrophils between severe asthma and control in technique B, in the percentage of lymphocytes between mild asthma and control in technique A and in the percentage of macrophages between moderate and severe asthma and control in technique A. In addition, there was no significant difference in the percentages of neutrophils, lymphocytes, and macrophages when the three techniques were compared for both control and asthmatic patients.

In order to study the correlation among the explored techniques, the Spearman rank correlation was applied to eosinophil and neutrophil counts, since they are more characteristic of airway inflammation in asthma. Eosinophil percentages in sputum studied by techniques A and C, and techniques B and C were closely correlated (r = 0.64 and r = 0.63, respectively; p < 0.01). There was also a positive correlation for eosinophil counts in the sputum between techniques A and B (r = 0.57; p < 0.01). The correlation of the percentages of neutrophils in the sputum was also significant when each technique was compared to the others (technique A vs technique B, r = 0.66; technique A vs technique C, r = 0.51; technique B vs technique C, r = 0.57; p < 0.01).

Bland-Altman summaries are shown in Figure 3. The agreement between the different techniques is demonstrated by a plot of differences against mean values of cell percentages. In this plot, solid bars represents the mean difference and broken lines indicate ± 2 SD from the mean. The various measures were reproducible since the individual points on the Bland-Altman plots were randomly scattered around the overall average difference and most.
points fell within 2 SDs of the overall difference. A random scatter with several points outside the 2 SDs would indicate a weak agreement.

The mean total time required for processing the sample and performing a differential cell count was 32 min for technique A, 38 min for technique B, and 66 min for technique C ($p < 0.001$). The costs in US dollars of the material used for each slide were estimated to be $4.40, $2.38, and $1.64 for techniques C, B and A, respectively.

**DISCUSSION**

In the present study, we examined the correlation and agreement of three different methods of processing IS from patients with mild, moderate, and severe asthma, and subjects without asthma. We measured the degree of correlation among the methods, and computed the costs of slide preparation and the time spent by the technicians. Popov et al\(^1\) proposed a new method of storing sputum samples, while Gibson et al\(^2\) elected to simplify sputum analysis. Instead of preparing cytospin specimens to quantify eosinophils, the most extensively validated cellular component in sputum, they lysed the cell pellet after homogenization and measured the amount of eosinophilic cationic protein released as a marker of the number of eosinophils present in sputum. Popov et al\(^3\) compared cytospin with sputum smear slides for differential cell counts and immunochemical staining in a blinded mode. The cells on the cytospin preparations were well dispersed without the clumps often seen on smears, and cell definition was much better. The time taken to count cells on cytospin specimens was significantly less than on smears. There was lower reproducibility of smear counts by the same examiner after repeated readings of the slides compared to cytospin preparations. Cytospin use resulted in good dispersion and definition of cell types and good reproducibility of neutrophil, eosinophil, and macrophage cell counts.

In our study, technique A (smear slides) showed clumps that sometimes impaired cell differentiation and count. Clumping of cells could be a potential source of error; however, after adequate training, this difficulty became less important. The smear is rich in cells, since the sample is not diluted, and it was always possible to avoid areas of cell clumping and perform the differential cell count. Clumps in the smears were avoided almost completely by pretreatment of the sputum with DTT before preparation. The proper identification of the different cell types was possible.

Figure 1. Top left, A: Representative slides of IS; from left to right, simple smear (technique A), sputum treated with DTT and smeared (technique B), and cytospin preparation (technique C). Also shown are photomicrographs of slides of IS processed by technique A (top right, B), technique B (bottom left, C), and technique C (bottom right, D) [Leishman, stain original, × 1,000].

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types was sometimes more difficult when using technique B compared to technique A, since we observed that cells tended to be smaller than with technique A and that there were more cellular debris than with technique C, since there was no filtration of the sample. The best resolution was obtained with cytospin preparations; however, our results indicate that the two simpler procedures provided the same basic results.

The agreement among the techniques was estimated by the Bland-Altman method using eosinophil and neutrophil counts as estimators. Figure 3 shows that the techniques were comparable because the individual points on the Bland-Altman plot are randomly scattered around the overall average difference and most points fall within 2 SDs of the overall average difference.

In our study, all techniques disclosed significant differences in cell counts between control subjects and patients with severe asthma (Fig 2). In all the three techniques used, all subjects with asthma had >3% eosinophils in the sputum, whereas all control subjects had <1%. Several previous investigations firmly established the occurrence of a high percentage of eosinophils in the sputum of asthmatic patients and that anti-inflammatory treatment reduces sputum eosinophilia, although even stable asthmatics still show an increase in the percentage of eosinophils in sputum compared to people without airway inflammation. Our study also showed a trend to higher percentages of eosinophils in the sputum of people with more severe asthma (Fig 2). Louis et al also observed a positive correlation between sputum eosinophilia and asthma severity. We did not observe a consistent difference in the percentage of neutrophils in the sputum of asthmatics compared to nonasthmatics. In a previous study, an increase in neutrophils in sputum has been observed during exacerbations of asthma. In contrast, Iredale et al and Fahy et al did not observe a significant difference in the percentage of neutrophils in the sputum of stable asthmatics compared to nonasthmatics.

Our results confirm the potential of IS examinations to monitor or optimize the treatment of asthmatic patients. We hope that the information provided by the present study—that comparable results could be ob-

Figure 2. Percentages of cells obtained from samples of IS from healthy subjects, and patients with mild, moderate, and severe asthma. Each sample was analyzed by techniques A, B, and C. Boxes represent the 25th to 75th percentiles, the line inside the boxes represents the median, and the bars represent the 10th and 90th percentiles. Top left, A: Percentage of eosinophils, *p < 0.05 compared to control, †p < 0.05 compared to control and mild asthma. Top right, B: Percentage of neutrophils, *p < 0.05 compared to control. Bottom left, C: Percentage of lymphocytes, *p < 0.05 compared to control. Bottom right, D: Percentage of macrophages, *p < 0.05 compared to control, †p < 0.05 compared to control and mild asthma.
tained with simplified techniques—will encourage further application of IS in routine practice.

Cytospin centrifugation is a refined method that allows cell differentiation and is more suitable for research since it permits the measurement of soluble mediators as well as immunostaining. Technique B could be successfully used for cell differentiation, also allowing the estimation of total cells in sputum using a Neubauer camera. Technique A can be easily applied in office settings, requiring only two slides, stain, and microscope, and could be used to monitor asthma treatment. However, although this is a very promising approach, this method needs to be validated before it can replace the standard existing cell dispersion methods. Cytospin could be used more for research purposes or in more specialized centers, and techniques A and B, which are faster and less costly, could be used in clinical practice.

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REFERENCES

Figure 3. Bland-Altman analysis comparing techniques A and B to the standard and accepted technique (technique C) [A-C and B-C, respectively]. The agreement of eosinophils (EOS) and neutrophils (NEUT) counts was studied. Solid lines represent the means of the differences (Diff) in individual values obtained by the two techniques; dashed lines represent 2 SDs.