

BIOMARKERS

Reproducibility of Sputum Differential Cell Counts Is not Affected by Squamous Epithelial Cells

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Objective. Induced sputum is used to assess markers of inflammation in asthmatic individuals, and sputum cell differential counts provide an outcome to evaluate the presence, type, and degree of inflammation in the airways. Contamination of sputum slides with squamous epithelial cells (SECs) has been reported to adversely affect the reproducibility of sputum cell differential counts; however, this has not been studied in a controlled manner. Excluding sputum slides because of excessive squamous cell contamination can be problematic resulting in under-powering of studies. The aim of this study is to evaluate the effect of SEC contamination and cell dispersion on the reproducibility of differential counts of sputum cells prepared on glass slides. **Study Design.** A total of 33 sputum samples were induced from 11 subjects with mild asthma under baseline conditions and following an allergen inhalation challenge. Mucoid and salivary portions of each sample were divided and processed in parallel. To evaluate the effect of increasing the proportion of SEC and to evaluate the effect of increasing the number of leukocytes per high power field (HPF), four slides with varying leukocyte numbers and SEC percentages were prepared from each sample by combining and adjusting the volume of cell suspensions derived from mucous and saliva. The four slides were prepared to fall in the following categories: (A) 50 cells/HPF and <20% SEC; (B) 50 cells/HPF and >20% SEC; (C) 100 cells/HPF and <20% SEC; and (D) 100 cells/HPF and >20% SEC. All slides were blinded and counted twice by an experienced observer, and twice by an inexperienced observer. **Results.** The differential cell counts for eosinophils, macrophages, and neutrophils were highly reproducible under all conditions when enumerated by an experienced observer (ICC > 0.9), and furthermore, SEC contamination did not affect ICC when differential counts were enumerated by an inexperienced observer (ICC > 0.8). **Conclusion.** Our results demonstrate that slides containing SECs, up to 40% in this study, have reproducible differential cell counts.

Keywords asthma, eosinophils, intraclass correlation, macrophages, neutrophils, reproducibility

INTRODUCTION

Differential cell counts from samples of induced sputum have been promoted for many years as a method of obtaining measurements of airway inflammation noninvasively. Examination of induced sputum from asthmatic patients is a very useful tool for clinical management of disease (1–4) and a powerful research outcome to assess disease severity and treatment efficacy (5–7). Sputum induction is reported to be successful in 80–90% of adult patients (8); however, the definition of “success” is still not well established and can vary depending on the quantity and quality of sample required for analyses.

Sputum samples are commonly rejected in clinical laboratories if there is an abundance of squamous epithelial cells (SECs), which are found in saliva and originate from the lining of the mouth. However, it is controversial whether the presence of SECs affects the accuracy and reproducibility of lower airway inflammatory cell counts. A large number of studies have contributed to sputum induction and processing guidelines published by the European Respiratory Society Task Force in 2002. One of these guidelines reported that the contamination of

expectorated sputum with saliva will adversely affect the reproducibility of differential leukocyte counts (9) and this has been widely referenced (10). This guideline, however, did not take into consideration published reports demonstrating that different percentages of SEC contamination in sputum samples has no effect on the reproducibility of eosinophil counts (11,12) which was confirmed several years later by Simpson et al. (13). A retrospective study by Ward et al. (12) had demonstrated numerically lower ICC values from samples with low cell viability and high (>20%) SEC; however, the authors concluded that the reproducibility of eosinophil counts was acceptable, and that no samples should be excluded from eosinophil analysis due to high SEC levels. Thus, the published recommendation by the ERS Task Force regarding SEC contamination in sputum samples is not consistent with the literature; however, the recommendation continues to be followed in clinical laboratories.

The importance of establishing updated evidence-based guidelines for acceptable levels of SEC contamination of sputum samples is important for several reasons. Clinically, sputum samples can be used to help in the diagnosis of asthma (14), prevention of exacerbations (15), or assess efficacy of therapy (16, 17). Discarding these samples and/or rescheduling the patient will prevent valuable and timely information from being collected. In longitudinal or cross-over designed studies with repeated

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measurements within subjects, statistical analyses are extremely sensitive to missing or excluded samples. In order to establish guidelines which reflect acceptable SEC contamination of sputum samples, this systematic study examined the effects of increasing SEC and decreasing dispersion of leukocytes on the reproducibility of differential leukocyte counts in sputum samples.

MATERIALS AND METHODS

Participants and Study Design

A total of 33 sputum samples were obtained from 11 subjects with mild allergic asthma. Subject demographics are shown in Table 1. Subjects were male and female, nonsmokers, with baseline forced expiratory volume in 1 second (FEV₁) >70% of predicted, methacholine provocative concentration causing a 20% fall in FEV₁ (PC₂₀) ≤16mg/ml, and a documented fall in FEV₁ >20% upon inhalation of an aeroallergen. The study was approved by the Hamilton Health Sciences/McMaster University Research Ethics Committee and informed consent was signed by all subjects. Samples were collected before and after (7 h and 24 h) allergen inhalation challenge when eosinophil levels peak in the sputum (5), thereby ensuring a wide range of eosinophil numbers in order to assess the reproducibility of eosinophil differential cell counts in sputum. Of the 33 samples collected, 30 samples yielded sufficient cell numbers for the experiments described. The sample size was considered sufficient for this study since comparisons were made on the degree of relatedness between counts of the prepared slides.

Sputum Induction and Processing

Sputum was induced and processed using validated methods (18, 19). Participants were treated with 200 µg inhaled salbutamol and 10 min later they inhaled 3%, 4%, and 5% saline in sequence for 7 min each. After each inhalation, subjects were asked to blow their noses and rinse their mouths and to expectorate into a sterile container. The sputum mucus plugs and the salivary portion of the samples were separated and processed in parallel. Cells were liquefied using 0.1% dithiothreitol,

and filtered through a 52 µm nylon gauze to remove debris. The viability and number of leukocytes were determined using Trypan blue exclusion and a Neubauer hemacytometer chamber. Cells from the mucoid portion and the salivary portion were pelleted by centrifugation at 500 × g for 10 min, then resuspended in Dulbecco's phosphate-buffered saline at a concentration of 1 × 10⁶ cells/ml. Cytospin preparations were made on glass slides using 50 and 100 µl of cell suspension and a Shandon III cytocentrifuge at 10 × g for 5 min. Four cytospin slides were created with varying leukocyte and SEC numbers by combining and adjusting the volumes of each cell suspension to achieve (A) 50 leukocytes/high power field (HPF) with <20% SEC; (B) 50 leukocytes/HPF with >20% SEC; (C) 100 leukocytes/HPF with <20% SEC; and (D) 100 leukocytes/HPF with >20% SEC. Slides were stained with Diff-Quik and 400 leukocytes per slide were enumerated by an experienced observer (>100 h sputum cell counting experience). The slides were also counted by an inexperienced observer who had never performed differential cell counting, and completed 1 h of training. Slides were re-randomized and counted a second time by each observer for the determination of within-observer ICC. An independent observer determined the number of leukocytes/HPF and the %SEC on each slide using a light microscope. The percentage of squamous cells in each sample was enumerated by a third observer from the cell suspension using a hemacytometer, and also from a stained glass slide during a separate count of SEC versus leukocytes.

Methacholine PC₂₀

Methacholine PC₂₀ was measured as described by Cockcroft et al. (20) using tidal breathing from a Wright nebulizer. Doubling concentrations of methacholine were administered until a ≥20% fall in FEV₁ of the baseline value occurred.

Allergen Inhalation Challenge

Allergen inhalation was performed as described by O'Byrne et al. (21) using the concentration of allergen extract determined from a formula described by Cockcroft and coworkers (22). Doubling concentrations of allergen were administered until a ≥20% fall in FEV₁ at 10 min post-allergen was reached.

Statistical Analysis

Descriptive summary statistics are expressed as mean ± standard deviation. The reproducibility of differential counting was evaluated for eosinophils, neutrophils, and macrophages for each observer using a two-way random effects model of intraclass correlation coefficient. The reproducibility of less common cells such as lymphocytes and basophils was not assessed. Analysis was performed using Predictive Analytics SoftWare Statistics version 18.0 (IBM, New York, NY, USA).

TABLE 1.—Subject demographics.

Subject	Age	Gender	Baseline Methacholine PC ₂₀ (mg/ml)	Baseline FEV ₁ (% predicted)
1	60	M	11.6	103
2	49	M	7.13	99
3	44	F	1.14	90
4	22	F	4.2	96
6	22	M	2.44	82
7	22	F	1.19	93
8	22	M	1.41	117
9	21	M	5.1	121
10	21	M	10.5	104
11	21	M	6.3	101

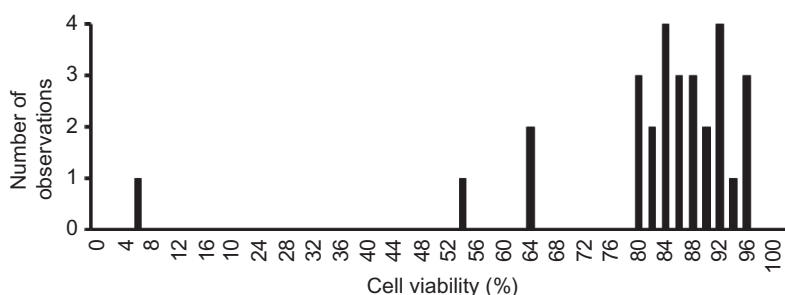


FIGURE 1.—Histogram showing the number of sputum samples within each range of viability.

RESULTS

Thirty sputum samples yielded sufficient mucous and saliva to prepare all slides. The average viability of leukocytes in these sputum samples was $81.1 \pm 17.4\%$; four of the samples had viability $<65\%$ (Figure 1). All samples were included in the analysis, regardless of cell viability in order to provide a realistic background to assess SEC. Since the cell viability was consistent across the four slide preparations studied, viability was not examined nor manipulated. The mean leukocyte number/HPF ($40\times$) and SEC/HPF on sputum slides for each of the four slide preparations is shown in Table 2. Representative sputum slides showing cell distribution of each of the four slide preparations are shown in Figure 2.

The reproducibility of differential counts for eosinophils, neutrophils, and macrophages under four different conditions, expressed as ICC, is shown in Table 3. The overall reproducibility for each condition had ICC > 0.9 when enumerated by the experienced observer. Of note, increasing SEC to 40% on slides with good cell dispersion (condition B) returned a high ICC of 0.97 for eosinophils (Figure 3), and 0.99 for both neutrophils and macrophages. The ICCs were lower when slides were enumerated by the inexperienced observer and fell below 0.8 when the leukocyte numbers increased greater than 50 leukocytes/HPF (conditions C and D).

The squamous cells were enumerated in the cell suspension during sample processing, and again on the glass slides after cells had been stained. We found that the proportion of SEC measured by hemacytometer was significantly lower than the proportion measured by differential counting on the glass slides ($p < .05$) (Figure 4).

TABLE 2.—Actual percentage of squamous epithelial cells and leukocytes/HPF ($40\times$ magnification) for each of the four slide conditions.

	A	B	C	D
Leukocytes (#/HPF)	43 ± 12	49 ± 13	91 ± 22	85 ± 20
SEC (#/HPF)	8 ± 6	19 ± 6	15 ± 11	2 ± 10
SEC (%)	17 ± 14	40 ± 11	17 ± 13	26 ± 11

Notes: (A) 50 leukocytes/HPF with $<20\%$ SEC; (B) 50 leukocytes/HPF with $>20\%$ SEC; (C) 100 leukocytes/HPF with $<20\%$ SEC; and (D) 100 leukocytes/HPF with $>20\%$ SEC. HPF, high powered field; SEC, squamous epithelial cells.

DISCUSSION

The reproducibility of cell differentials was examined using sputum cell cytopspins prepared from the same sample, but differing in cell dispersion and proportion of SECs. The reproducibility of cell differentials was assessed by intraclass correlation; ICCs were found to be greater than 0.9 for eosinophils, neutrophils, and macrophages under each of the four conditions when enumerated by an experienced observer. This includes slides having SEC 20–40%.

The ICCs for the sputum cell counts performed by the inexperienced observer ranged from 0.80–0.85 when the cell dispersion was 50 leukocytes/HPF, and SEC contamination was minimized (condition A). The ICC was not adversely affected with the introduction of higher SEC contamination, with ICCs ranging from 0.83 to 0.90 in condition B. The ICCs were consistently lower than those of the experienced observer under conditions C and D when the number of leukocytes/HPF increased. This suggests that overcrowding of cells on the slide rather than SEC contamination is a factor contributing to poor reproducibility of cell counts.

The findings suggest that the reproducibility of differential cell counting by experienced observers is excellent in sputum samples having SEC $> 20\%$, despite reports in the literature that SEC leads to more variable counts (9). Surprisingly, even the ICC values calculated from the inexperienced observer were high, being 0.90 for eosinophils in samples having SEC $> 20\%$. These data, regardless of the observer, do not support the ERS Task Force guidelines which suggest low reproducibility of differential cell counts in sputum samples with SEC $< 20\%$, albeit referencing only a conference abstract. In this study, appropriate cell dispersion was maintained to avoid overlapping of cells, and the cell viability of most sputum samples was $>80\%$. With the less-experienced observer, the cell dispersion appears to have a greater impact on reproducibility of cell differentials, as reflected by consistently lower ICC values obtained for conditions C and D. This study demonstrates ICC values similar to those previously reported by Spanevello et al. (23) and Pizzichini et al. (19) which showed good repeatability within sample and between examiners for eosinophils (0.98 and 0.99, respectively), macrophages (0.86 and 0.97, respectively), and neutrophils (0.85 and 0.98, respectively). It is

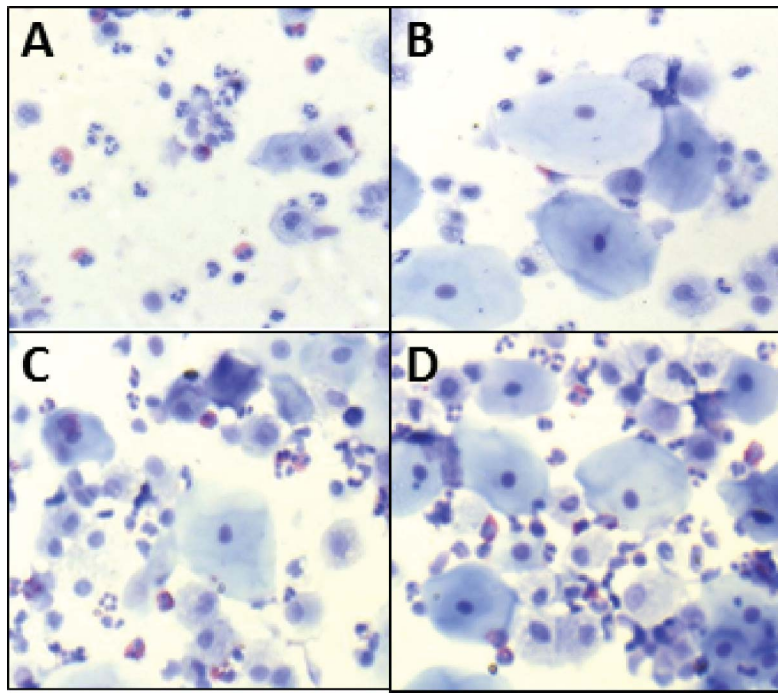


FIGURE 2.—Sputum cytopspins from a representative sample with varying leukocyte numbers and squamous epithelial cells.

TABLE 3.—Intraclass correlation coefficient for each of the four slide conditions, enumerated by an experienced and an inexperienced observer.

	Experienced observer				Inexperienced observer			
	A	B	C	D	A	B	C	D
Eosinophils	0.98	0.97	0.98	0.94	0.85	0.90	0.86	0.76
Neutrophils	0.99	0.99	0.97	0.97	0.80	0.83	0.73	0.68
Macrophages	0.91	0.99	0.98	0.99	0.83	0.90	0.73	0.69

Notes: (A) 50 leukocytes/HPF with <20% SEC; (B) 50 leukocytes/HPF with >20% SEC; (C) 100 leukocytes/HPF with <20% SEC; and (D) 100 leukocytes/HPF with >20% SEC. HPF, high powered field; SEC, squamous epithelial cells.

noteworthy that in these previous studies, the assessments were conducted with slides having a much lower %SEC of 4.8% and 1.2%, respectively. These comparable ICC values clearly suggest that the higher level of SEC on our cytopspins does not deleteriously affect the ability to carry out a reproducible differential cell count in induced sputum.

We have demonstrated that appropriate cell dispersion on the cytopspins is important for reliable sputum cell differential counts, as the ICC was lower for slides with higher numbers of cells per HPF. Since SECs are much larger than the leukocytes, a relatively small percentage of SEC can result in suboptimal cell dispersion due to overlapping of cells. The proportion of SEC measured by hemacytometer during sputum sample processing is significantly lower than the proportion of SEC on cytopspins, which may be an artefact introduced by preferential deposition of large cells during cytocentrifugation. This is important to note because our data suggest inadequate cell dispersion can negatively impact the reproducibility of differential cell counts.

This is the first study designed with the goal to systematically examine the impact of SEC and cell

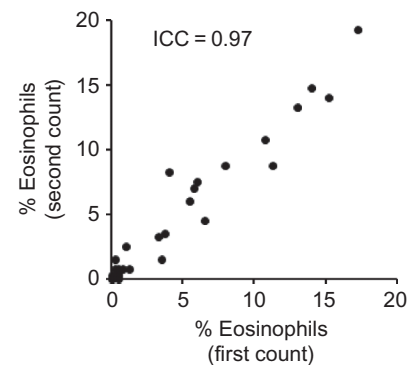


FIGURE 3.—Intraclass correlation for sputum eosinophils counted twice by an experienced observer, on slides prepared according to condition B (50 leukocytes/high power field with >20% squamous epithelial cells).

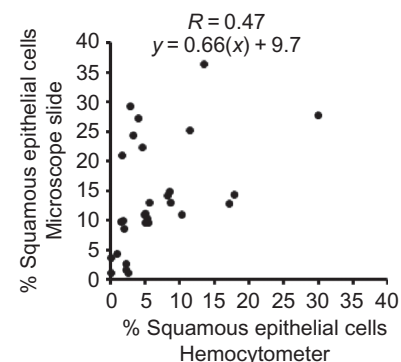


FIGURE 4.—The relationship between squamous epithelial counts in sputum samples by hemacytometer versus differential cell counting.

dispersion on the reproducibility of sputum cell differential counts. This study was limited by the inability to observe a broad range in cell numbers for each population studied. Although the sputum samples were obtained from subjects with mild asthma who have a relatively normal sputum differential profile with high numbers of macrophages (24), many of the samples were collected post-allergen challenge when the number of eosinophils and neutrophils were elevated. This enabled a wider range of each cell population for the calculation of accurate ICC values. We were unable to assess reproducibility of lymphocytes or basophils in this study, since they are rare cell types in sputum and would require a more specific stain for ease of identification. Despite these limitations, our results suggest that differential counts of eosinophils, neutrophils, and macrophages prepared from induced sputum samples containing high levels of SECs can be enumerated reproducibly.

CONCLUSIONS AND KEY FINDINGS

Our findings conclude that sputum samples having SEC contamination as high as 40% can be enumerated with excellent reproducibility. That SEC does not negatively impact sputum differential counts is not a surprising finding; however, this study provides essential evidence which will permit much more efficient use of clinical specimens.

DECLARATION OF INTEREST

None of the authors have any financial, consulting, or personal relationships with other people or organizations that could influence the work reported in this manuscript. There was no scientific writing assistance utilized. ZS, BM, and SGS are graduate students at McMaster University, and TS, AT, KH, and GG are employed by McMaster University.

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