A method to preserve sputum for delayed examination

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A method to preserve sputum for delayed examination. M.M. Kelly, F.E. Hargreave, G. Cox. ©ERS Journals Ltd 2003.

ABSTRACT: Examination of sputum cell counts is limited by the need to process samples within hours of expectoration. The validity and repeatability of a method to preserve sputum for delayed processing and examination were investigated in this study.

Portions of selected sputum from 39 subjects were dispersed with dithiothreitol (routine method), or were fixed in a dithiothreitol-formaldehyde mixture for 48–72 h before dispersal with trypsin (preservation method). Total and differential cell counts using the two methods were compared; within-method repeatability of the preservation method was also examined.

The intraclass correlation coefficient (ICC) for total cell counts and percentage of eosinophils, neutrophils and macrophages in sputum processed by the two methods was 0.89, 0.86, 0.91 and 0.90, respectively. Within-method repeatability (ICC) of the preservation method for the same cellular indices was 0.99, 0.94, 0.97 and 0.97, respectively. The interobserver repeatability for eosinophils, neutrophils and macrophages was 0.96, 0.97 and 0.97 using the preservation method, and 0.96, 0.99 and 0.99 using the routine method, respectively.

This method of sputum preservation and dispersal is valid, reliable and convenient, and may be used for delayed processing and examination.

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The use of induced sputum examination has been developed as a noninvasive, valid and reliable research tool to objectively assess the presence, nature and severity of airway inflammation in asthma and other conditions [1–5]. It has been suggested that the method can be used to monitor airway inflammation and provide information to aid in decisions about treatment requirements [6–8]. However, the widespread clinical application of induced sputum examination is limited by the need to process it on the day of expectoration (≤ 9 h delay at 4°C [9]), and for appropriate equipment and suitably trained technicians to be readily available. The technique of SACCOMANNO *et al.* [10], a method that has been developed in an attempt to allow delayed processing of sputum, results in a 24% reduction in cellularity and poor cell morphology when compared with homogenisation of fresh sputum with dithiothreitol (DTT) [11, 12]. It was hypothesised that sputum, being viscous, should be fixed with a rapidly penetrating fixative, which would also serve to inhibit intracellular lytic enzymes [13, 14]. Paraformaldehyde, a cross-linking fixative, has a rapid action, and avoids the shrinkage seen when dehydrating fixatives, such as alcohol are used [15]. Paraformaldehyde-fixed tissue can be successfully dispersed by proteases to obtain single cell suspensions for flow cytometry [16, 17] and *in situ* hybridisation [18–20]. Dispersal of fresh sputum with trypsin has been described previously [21], but prior fixation allows the cells to withstand this relatively harsh treatment. In a series of pilot studies, fixation with varying concentrations of paraformaldehyde and DTT, and dispersion with trypsin, protease XIV and chymotrypsin were examined.

On the basis of these studies, a prospective study was conducted, where a portion of sputum in a paraformaldehyde-DTT mixture was preserved and dispersed with trypsin

(preservation method). The cell counts obtained were then compared with those from a portion of the same sputum that was immediately processed with DTT [3] (routine method). The aim was to develop a method that allows delayed processing and examination of sputum samples.

Materials and methods

Subjects

Sputum samples from 39 patients (16 male, mean age 46.3 yrs) attending the Firestone Institute for Respiratory Health were examined. Thirty-five patients had asthma, two had chronic cough, one had chronic obstructive pulmonary disease and one had cystic fibrosis. The study protocol was approved by the Hospital Research Ethics Board, and all patients provided written informed consent to participate in the study.

Study design

This was a prospective, cross-sectional study in which induced sputum samples from 39 patients were randomly divided into two portions; in 27 of the 39 patients an additional third portion was also obtained. One portion was processed by the routine method, while the other portion(s) were processed by the preservation method. Total and differential cell counts were compared between the two methods and within the preservation method. All samples were coded and sputum cell counts were performed blind to clinical details. The same technologist examined the total cell counts on all samples

from the same subject and differential cell counts were performed by one technologist. To test between-observer repeatability of the method, another technologist read the coded slides processed by the routine and preservation methods.

Procedures

Sputum induction. Sputum was induced as described by PIN *et al.* [1] and modified by PIZZICHINI *et al.* [3] except in two cases where it was spontaneously expectorated.

Sputum processing. Sputum was selected from the expectorate as described by PIZZICHINI *et al.* [3] within 2 h of collection, divided into two or three approximately equal portions (depending on the volume available), and randomly assigned to be processed by the routine method (one portion) or the preservation method (one or two portions) as shown in figure 1.

Routine method. This consisted of the addition of four volumes of freshly prepared 0.1% DTT (Sputolysin; Calbiochem, La Jolla, CA, USA) and further processing was as described by PIZZICHINI *et al.* [3]. A differential cell count of cytopins stained by Wright's stain was performed by counting 400 nonsquamous cells.

Preservation method. The sample was immersed in 10 mL of a freshly prepared solution of 2% paraformaldehyde (ICN, Montreal, QC, Canada) and 0.1% DTT. The sputum mixture was briefly vortexed and then kept at room temperature for 48–72 h, after which the fixative removed. The sputum was washed three times by suspending in phosphate-buffered saline after centrifugation (300×g for 10 min), before adding a

solution (3 v/w, working concentration ~2%) of 2.5% trypsin (Sigma, Mississauga, ON, Canada; 0.05 M tris-buffered saline with 0.1% calcium chloride). After vortexing, the solution was incubated at 37°C for 6–17 h, depending on whether it was more convenient to complete the process on the same day or after an overnight incubation. The dispersed sputum was then briefly vortexed, filtered, the total cell count determined and cytopins prepared and stained with Wright's stain. The slides were then coded and differential cell counts performed on 400 nonsquamous cells. Viability of the fixed cells could not be determined.

Statistical analysis

The study was designed to have >80% power to detect a minimally important difference in total cell counts of 3.5×10^6 , with an alpha specification of 0.05, assuming a standard deviation of 7×10^6 . Sputum cell counts were expressed as median values and interquartile range (IQR). Comparisons between cell counts were made by the Wilcoxon-signed ranks test for paired data. Bland-Altman graphs and statistics were performed as described in BLAND and ALTMAN [22]. Reproducibility was expressed by intraclass correlation coefficients (ICC) as the ratio of variance among subjects to total variance with the 95% confidence limits. Two-tailed p-values <0.05 were considered significant.

Results

The total and differential cell counts obtained by the routine and preservation methods were closely correlated (table 1 and fig. 2a). Significantly higher total cell counts of eosinophils and neutrophils (absolute and percentage) were observed when samples were processed with the preservation method compared with the routine method (table 1 and fig. 3). However, despite the proportion of macrophages being reduced, the absolute macrophage cell counts were unchanged, indicating that the increased total cell counts were due to a real increase in eosinophils and neutrophils. There was no correlation between the length of time of fixation or of dispersal of the preserved sample and the difference in cell counts between the methods.

Table 2 shows the cell counts obtained when a second portion was processed by the preservation method, indicating good repeatability and no significant differences from that of the cell counts in the first portion processed by the same method (figs 2 and 4). The between-observer repeatability of the routine and preservation methods was high (table 3).

When 1.1 was used as the upper limit for per cent eosinophil counts in normal subjects with the routine method [23], the preservation method correctly identified all but three samples as being above or below this limit; the difference

Fig. 1.—Illustration of sputum processing, division of aliquots and treatment by routine or preservation methods. DTT: dithiothreitol; PBS: phosphate-buffered saline.

Table 1.—Comparison of cellular indices in sputum processed by the routine and the preservation methods

	Routine method	Preservation method	p-value [#]	ICC (95% CL)
TCC $\times 10^6$ cells·g ⁻¹	4.8 (7.2)	8.4 (9.4)	<0.001	0.89 (0.78–0.94)
Eosinophils %	2.3 (8.1)	3.2 (14.8)	<0.05	0.86 (0.72–0.93)
Eosinophils $\times 10^6$ cells·g ⁻¹	0.21 (0.56)	0.37 (1.13)	<0.001	0.65 (0.32–0.82)
Neutrophils %	32.4 (43.5)	45.4 (53.6)	<0.05	0.91 (0.82–0.95)
Neutrophils $\times 10^6$ cells·g ⁻¹	1.46 (2.82)	4.26 (8.25)	<0.001	0.95 (0.91–0.98)
Macrophages %	59.7 (51.5)	42.3 (48.2)	<0.001	0.90 (0.8–0.95)
Macrophages $\times 10^6$ cells·g ⁻¹	1.88 (2.65)	2.08 (2.1)	NS	0.88 (0.77–0.94)

Data are presented as median (interquartile range) unless otherwise stated. ICC: intraclass correlation coefficient; CL: confidence limit; TCC: total cell count. NS: nonsignificant. [#]: Wilcoxon-signed rank test.

Fig. 2.—Bland-Altman plots of differences against mean values of total cell counts (TCC) show the agreement between samples processed by the two different methods: a) total sputum cell counts from aliquots from the same sample processed by the routine and preservation methods (mean difference, 95% confidence interval (CI) for lower and upper limits of agreement are 3.6, 4.3 and 8.5, respectively), and b) two aliquots both processed by the preservation method (mean difference, 95% CI for lower and upper limits of agreement are 0.6, 1.3 and 2.9, respectively). The solid line represents the mean difference, and the dotted lines indicate ± 1.96 SD from the mean *i.e.* the 95% limits of agreement.

between the routine and preservation methods for these three samples was ≤ 1.8 . Similarly, taking 64.4 as being the upper limit for per cent neutrophil counts [23], the preservation method correctly identified all but four samples above or below this limit.

The cell viability (as measured with trypan blue exclusion in the routine method) was median (IQR), 62.6% (21), with seven samples having a viability of $< 50\%$; viability could not be measured with the preservation method. There was no correlation between the cell viability measured in this way and the difference in cell counts between the two methods. Squamous cell contamination was $< 20\%$ in all cases. The cell morphology in the routine and preservation methods is compared in table 4 and figure 5. There was no correlation between cell viability of the sample and what slides were rejected because of poor morphology.

In general, the routine method produced slides with a clean background, while in the preservation method there was background deposit of varying quantity, which appeared to consist of preserved necrotic debris, although this deposit did not interfere with cell classification.

Discussion

This study compared total and differential cell counts in sputum processed on the same day with DTT (routine method), with those in sputum processed after a delay of 48–72 h (preservation method), and showed good agreement between the two techniques as assessed by the ICC. This is the first time a method has been described that allows for delayed processing of sputum without cell loss and with preserved differential cell counts. Despite the preservation method resulting in higher numbers of eosinophils and neutrophils overall, there was close agreement between the two methods in identifying what samples had per cent eosinophil and neutrophil counts above or below the 90th percentile for normal subjects.

The within-method repeatability of the preservation method for all cell counts was high, with narrow confidence intervals. In addition, the between-observer repeatability of the preservation method was similar to that of the routine method in this study, and to that published for the routine method (ICC

Fig. 3.—Differential cell counts (%) of sputum processed by the routine and preservation methods. The agreement between samples processed by the two different methods is demonstrated by Bland-Altman plots of differences against mean values for a) eosinophils (mean difference, 95% confidence interval (CI) for lower and upper limits of agreement are 3.4, 5.2 and 10.5, respectively), b) neutrophils (mean difference, 95% CI for lower and upper limits of agreement are 8.8, 10.6 and 21.5, respectively), and c) macrophages (mean difference, 95% CI for lower and upper limits of agreement are -11.3, 11.3 and 22.9, respectively). The solid line represents the mean difference, and the dotted lines indicate ± 1.96 SD from the mean *i.e.* the 95% limits of agreement. It should be noted that the difference between eosinophil counts increases as the counts increase, therefore the precision of the limits of agreement is underestimated for low eosinophil counts and overestimated for high eosinophil counts.

Table 2. – Comparison of cellular indices in two aliquots of sputum both processed by the preservation method

	Aliquot 1	Aliquot 2	p-value [#]	ICC (95% CL)
TCC $\times 10^6$ cells·g ⁻¹	8.8 (7.5)	9.6 (11.0)	NS	0.99 (0.98–0.99)
Eosinophils %	2.5 (12.2)	4.0 (11.0)	NS	0.94 (0.84–0.98)
Eosinophils $\times 10^6$ cells·g ⁻¹	0.44 (0.89)	0.44 (1.11)	NS	0.91 (0.73–0.97)
Neutrophils %	50 (59.7)	57.7 (64.9)	NS	0.97 (0.93–0.99)
Neutrophils $\times 10^6$ cells·g ⁻¹	6.03 (12.88)	6.31 (13.48)	NS	0.99 (0.97–0.99)
Macrophages %	24 (62.3)	12.3 (63.3)	NS	0.97 (0.91–0.99)
Macrophages $\times 10^6$ cells·g ⁻¹	2.16 (3.27)	1.1 (2.03)	NS	0.88 (0.66–0.96)

Data are presented as median (interquartile range) unless otherwise stated. ICC: intraclass correlation coefficient; CL: confidence limit; TCC: total cell count. NS: nonsignificant. [#]: Wilcoxon-signed rank test.

Fig. 4. – Differential cell counts (%) of two aliquots of sputum both processed by the preservation method. The within-method agreement is demonstrated by Bland-Altman plots of differences against mean values for a) eosinophils (mean difference, 95% confidence interval (CI) for lower and upper limits of agreement are 0.7, 2.6 and 8.1, respectively), b) neutrophils (mean difference, 95% CI for lower and upper limits of agreement are -4.9, 4.8 and 14.9, respectively), and c) macrophages (mean difference, 95% CI for lower and upper limits of agreement are 4.6, 5.5 and 17.1, respectively). The solid line represents the mean difference, and the dotted lines indicate ± 1.96 SD from the mean, *i.e.* the 95% limits of agreement. It should be noted that the difference between eosinophil counts increases as the counts increase, therefore the precision of the limits of agreement is underestimated for low eosinophil counts and overestimated for high eosinophil counts.

Table 3. – Between-observer repeatability for the routine and preservation methods

	Eosinophils %	Neutrophils %	Macrophages %
Routine method	0.96 (0.86–0.97)	0.99 (0.98–0.99)	0.99 (0.97–0.99)
Preservation method	0.96 (0.89–0.98)	0.97 (0.86–0.97)	0.97 (0.94–0.99)

Data are presented as intraclass correlation coefficient (95% confidence limits).

Table 4. – Cell morphology quality in the routine and preservation methods

Quality of cell morphology	Routine method n	Preservation method n
Minimal degeneration	20	22
Moderate degeneration [#]	5	8
Severe degeneration [¶]	11	6
Unsuitable for analysis [‡]	3 [§]	3 [§]
Total suitable for analysis of differential cell counts	36	36

[#]: degenerate cell changes present but 400 nonsquamous cells can be counted; [¶]: numerous unclassifiable cells, only 100–200 nonsquamous cells can be counted; [‡]: severe cellular loss and disruption, and excluded from analysis; [§]: one sample was unsuitable with both the preservation and routine methods.

Fig. 5. – Comparison of cell morphology (Wright's stain) between a) the routine and b) the preservation method. E: eosinophils; N: neutrophils; M: macrophages. Scale bars=10 μ m.

0.90, 0.89 and 0.90 for percentage eosinophils, macrophages and neutrophils, respectively) [24].

DTT has been shown to reduce both cell viability [25, 26] and neutrophil recovery from sputum [26]. The significantly increased recovery of eosinophils and neutrophils with the preservation method may be due to the rapid inactivation of cellular enzymes and stabilisation of the cell membrane by paraformaldehyde.

The use of the preservation technique would allow sites lacking facilities for processing sputum to collect sputum and transport it days later to a central processing facility where it could be processed in batches, with overnight dispersal. It may be possible to extend the period of fixation to >72 h, since the sputum is probably fixed adequately after 24 h. Increased convenience and reduced costs could encourage more widespread use of sputum examination, benefiting patients, clinicians and researchers. This method does not replace the routine method that allows the measurement of cell viability as well as soluble mediators such as cytokines. It is recommended that only one method is used to monitor airway inflammation within a subject to prevent the introduction of bias.

The preservation method may have other applications, such as screening for lung cancer, since atypical and malignant cells are often difficult to detect in sputum smears. Methods of sputum dispersal to allow better visualisation of these often fragile cells may disrupt them, resulting in false negatives, and this could theoretically be prevented by the use of the preservation method.

To conclude, the authors have described a convenient method for preservation and elective dispersion of sputum, allowing for the collection of sputum in a primary care setting and its subsequent transportation to a specialised centre for processing. This would encourage more widespread use of sputum as a research and clinical diagnostic tool.

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