Effect of repeated sputum induction on cell counts in normal volunteers

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Abstract

Background – Sputum induction is becoming more commonly used to assess airway inflammation. Since it is a relatively non-invasive procedure it may be useful for repeated measurements over a short period of time.

Methods – To assess the repeatability of the method over a 24 hour period, eight healthy, non-smoking, non-atopic subjects (four men) of mean age 30 years underwent sputum induction, repeated at eight hours and 24 hours. Sputum was induced by inhalation of 3.5% saline. Absolute and differential counts of inflammatory cells were performed on the whole sputum after dilution in Hank's balanced salt solution containing 1% dithiothreitol to solubilise the mucus content of the samples.

Results – There was a significant rise in the percentage of neutrophils in the eight hour sample compared with the baseline (57%, range 25–94% at eight hours compared with 28%, range 19–60%: median difference 22%) which remained high in the 24 hour sample (76%, range 37–83%: median difference 26%). The median baseline percentage of macrophages was 55% (range 26–69%) which fell to 38% (range 3–56%: median difference 22%) at eight hours and 19% (range 14–59%: median difference 25%) at 24 hours. There was no significant change in the differential counts of eosinophils, lymphocytes or columnar epithelial cells, nor in any of the absolute cell counts, at any time point.

Conclusions – Sputum induction may have limited utility in serial assessment of neutrophilic airway inflammation in normal subjects within a 24 hour period.

(Thorax 1998;53:87–90)

Keywords: sputum induction, neutrophils, hypertonic saline.

Analysis of cells and mediators in the lung has traditionally used samples obtained by bronchoalveolar lavage (BAL). This technique has a number of disadvantages as a research tool. It can be difficult to recruit volunteers for bronchoscopy studies and bronchoscopy may not be considered safe in those with severe lung disease. BAL is an invasive procedure and this limits the number of times it can be repeated, which makes serial measurements over a short period of time difficult.

Recently, the technique of induction of sputum by inhalation of nebulised hypertonic saline has been developed. Initially used as a

diagnostic tool to investigate lung cancer and respiratory infection, it has become widely used in the investigation of inflammatory cells and mediators in normal subjects and in patients with asthma and chronic obstructive pulmonary disease (COPD). Recent studies have demonstrated the repeatability,^{1–3} responsiveness,^{4,5} and validity^{1–3} of this method. Sputum induction is quick, safe and allows repeated samples to be collected with relative ease.

In view of the increasing popularity of sputum induction, we undertook the present study to determine the repeatability of cell counts when three samples were collected at baseline (time 0 hours), eight, and 24 hours.

Methods

SUBJECTS

Eight subjects (four men) of mean (SE) age 29.9 (1.7) years were recruited. All were healthy with no history of respiratory illness and were non-smokers or ex-smokers of at least five years. All subjects had normal spirometric values (mean (SE) forced expiratory volume in one second (FEV₁) as percentage of predicted value 100.6 (3.3)%), a screening PC₂₀ to methacholine of >64 mg/ml and were non-atopic on skin prick testing to common aeroallergens (house dust mite, grass pollen, cat, and *Aspergillus fumigatus*). The study was approved by the ethics committee of the Royal Brompton Hospital and National Heart and Lung Institute.

STUDY PROTOCOL

Subjects underwent a screening visit including spirometric testing, measurement of PC₂₀ to methacholine, and skin prick testing to common aeroallergens. On a separate day subjects underwent sputum induction at three time points, the first in the morning, the second eight hours later, and the third the following morning 24 hours after the initial induction.

SPUTUM INDUCTION PROCEDURE

Subjects inhaled 3.5% saline for 15 minutes in total via an ultrasonic nebuliser (DeVilbiss 2000; DeVilbiss Co, Heston, UK) with a calibrated mass median aerodynamic diameter of 4.5 µm and the output set at 4.5 ml/min. The aerosol was inhaled through a tube 110 cm long with an internal diameter of 22 mm equipped with a mouthpiece, with the subject's nose clipped. Subjects discarded excess saliva into a separate bowl and mouthwashed thoroughly

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Received 1 August 1997
Returned to authors
14 October 1997
Revised version received
28 October 1997
Accepted for publication
18 November 1997

Figure 1 Effect of repeat sputum induction at 0 (baseline), eight and 24 hours on (A) neutrophils and (B) macrophages in eight normal subjects. Individual data points and medians (horizontal bars) are shown. Values at eight and 24 hours are significantly different ($p < 0.05$) compared with baseline values.

before each expectoration. Any secretions collected during the first five minutes were discarded in order to minimise squamous epithelial cell contamination. Secretions expectorated over the next 10 minutes were saved for analysis. These samples are termed "sputum" throughout this paper. All sputum samples were kept at 4°C for not more than two hours prior to processing.

SPUTUM PROCESSING

The whole sputum sample was diluted with 1 ml Hank's balanced salt solution (HBSS) containing 1% dithiothreitol (DTT) (Sigma Chemicals, Poole, UK) and was gently vortexed at room temperature. When homogeneous, the volume was recorded and the sample was further diluted with HBSS and centrifuged at 300g for 10 minutes. The supernatant was separated and the cell pellet resuspended in 1 ml HBSS. Total cells were determined on a haemocytometer slide using the Kimura stain and slides were prepared using a cytopsin (Shandon, Runcorn, UK) and stained with May-Grunwald-Giemsa. Differential cell counts were performed by a blinded observer with 300 non-squamous cells counted on each of two slides for each sample. Differential cell counts were expressed as a percentage of lower airway cells – that is, excluding squamous epithelial cells. Percentage squamous cell counts were expressed as a percentage

of total cells. Samples were considered adequate for analysis if there was <50% squamous cell contamination.

ANALYSIS OF DATA

Data for absolute and differential cell counts are not normally distributed^{3,4} and are expressed as medians and ranges throughout. The magnitude of changes from baseline are derived for each subject according to the formula: (differential count at x hours) – (differential count at baseline). These values are then used to calculate the median change in differential counts. Data at different time points were initially analysed using the Kruskal-Wallis test. When a significant difference between groups was observed, comparisons were made using the Wilcoxon signed rank sum test between two predetermined groups – namely, baseline versus eight hours and baseline versus 24 hours. A p value of less than 0.05 was considered significant.

Results

All subjects tolerated the sputum induction procedure well and there were no adverse events during the course of the study. All subjects produced an adequate sample of sputum at all three time points.

CELL COUNTS

There was a significant rise in the percentage of neutrophils in the eight hour sample compared with the baseline (57%, range 25–94% at eight hours compared with 28%, range 19–60%: median difference 22%) which remained high in the 24 hour sample (76%, range 37–83%: median difference 26%) (fig 1A). The rise in neutrophils was accompanied by a significant fall in percentage macrophages from a baseline value of 55% (range 26–69%) to 38% (range

Table 1 Differential cell counts in induced sputum in eight normal subjects

Time of sputum induction	Squamous cells	Eosinophils	Lymphocytes	Epithelial cells
Baseline	24.3 (2.9–49.0)	0 (0–0.4)	1.0 (0.7–4.5)	3.3 (1.1–25.8)
8 hours	19.1 (5.4–50.0)	0 (0–0.4)	0.7 (0–2.4)	3.6 (0.9–19.5)
24 hours	27.8 (3.6–46)	0.1 (0–1.5)	0.4 (0–1.4)	3.1 (1.5–4.7)

Values are medians with ranges. Percentage squamous cells are expressed as a percentage of the total cell count. Differential counts for other cell types are expressed as a percentage of lower airway cells, excluding squamous cells.

3–56%: median difference 22%) at eight hours and to 19% (range 14–59%: median difference 25%) at 24 hours (fig 1B). There was no significant change in the differential counts of eosinophils, lymphocytes, or columnar epithelial cells at any time point (table 1). There was a tendency for a rise in the absolute neutrophil counts from a baseline median value of 0.4 million/ml (range 0.03–2.2 million/ml) to 0.7 million/ml (range 0.02–1.8 million/ml) at eight hours and 0.7 million/ml (range 0.09–2.7 million/ml), although this failed to reach statistical significance. There were no significant changes in the absolute numbers of the other cell types.

Discussion

The results of this study show that differential neutrophil and macrophage counts from induced sputum samples in normal subjects are altered when repeated at eight and 24 hours. This is consistent with a study by Holz *et al* demonstrating mean increases of 13–19% in differential neutrophil counts in induced sputum repeated 24 hours after a previous induction.⁶ In contrast, repeatability of cell counts in normal subjects has been demonstrated previously¹³ with intraclass correlation coefficients of >0.7 for all cell types except lymphocytes (0.3) and total cell counts (0.4). However, the latter studies differed from ours in two ways. Firstly, both looked at sputum samples collected at least 24 hours apart. Secondly, they used the method of sputum “plug” selection rather than the whole sputum method used in the present study. This reduces salivary content of the sample and should improve reproducibility of absolute cell counts by reducing the variable dilutional effect of salivary contamination. Theoretically, reproducibility of differential counts should be unaffected by plug selection since these results are expressed as a percentage of total lower airways cells, excluding squamous cells. However, both intra- and inter-observer error in cell counting increases with increasing squamous cell contamination.⁷ A method that decreases squamous contamination should therefore increase cell count accuracy. This is unlikely to be a major factor accounting for the differences seen in neutrophil and macrophage counts in the present study for two reasons. Firstly, the squamous cell counts are low (19–28%) (table 1) in comparison with other studies using a similar method,⁴ although higher than in studies using plug selection.⁸ Secondly, the median squamous contamination was similar in all three samples (baseline 24%; eight hours 19%; 24 hours 28%).

In previous studies in which the whole sputum method has been used,^{4,7} although repeatability has been demonstrated, there appears to be greater variability of differential neutrophil counts. In a study of repeatability where sputum samples were induced at least two days apart, the intraclass correlation for neutrophils was 0.6 compared with 0.8 in Pizzichini *et al*.³ It is not clear whether this discrepancy is due to methodological differences

or to a different population of subjects. In't Veen *et al*⁷ studied asthmatic subjects only whilst Pizzichini *et al*³ looked at healthy subjects as well as patients with asthma and bronchitis. In a study of repeatability in asthmatic subjects over a 20 hour period,⁴ although there was no increase in the median value of neutrophils between the two samples, there was an increase in the range in the second sample.

It is of note that all of the studies mentioned above, as well as the present study, have utilised differing methods of inducing sputum. These differences include not only the concentration of hypertonic saline used but also marked differences in nebuliser outputs and nebulisation times. In the present study we used a nebuliser output of 4.5 ml/min. This is higher than that of 0.87 ml/min used by Pizzichini *et al*³ and 2.4–2.5 ml/min used by others.^{4,7} It is possible that the increase in neutrophils seen in the present study with repeated sputum induction may not have occurred with a lower output nebuliser. Other factors such as saline concentration and duration of nebulisation could also influence repeated sputum cell counts and formal studies of the effects of all these factors are warranted.

The mechanism responsible for the increase in neutrophils with repeat sputum samples is not clear. It may be due to an effect of the hypertonic saline. Previous studies have shown that exposure of human airways to hypertonic saline causes release of histamine, prostaglandin E₂ (PGE₂), and leukotriene C₄ from mast cells.^{9–11} Further support for a role of histamine is provided by the finding that the bronchoconstriction produced by inhaled hypertonic saline can be inhibited by the mast cell stabilising drug sodium cromoglycate and by terfenadine, a histamine H₁ receptor antagonist.^{12,13} This suggests that hypertonic saline inhalation can cause increased airway inflammation via the release of mast cell mediators. It is not possible to say from our data whether the increase in differential neutrophil counts is due to an inflammatory effect of hypertonic saline. The fact that there is no statistically significant increase in the absolute neutrophil counts may argue against a pro-inflammatory effect of the saline, and further studies are required to address this issue.

If hypertonic saline is a pro-inflammatory stimulus then it might be argued that the inflammatory cells found in induced sputum are a reaction to the hypertonic saline rather than pre-existing in the airway lumen. If this is the case, samples obtained by sputum induction should contain a higher proportion of neutrophils than those collected by BAL or bronchial washings. It has been shown that neutrophil counts in asthmatic subjects are markedly higher in induced sputum (36%) than in bronchial washings (5%) or BAL fluid (1.3%).^{14,15} However, it is more likely that the differences between sputum and BAL fluid are a consequence of the examination of different compartments in the airways. Further evidence against an immediate inflammatory response to hypertonic saline is that differential counts in induced sputum do not differ from those in

spontaneously induced sputum.¹⁶ Also, there is no difference in differential cell counts from sputum induced by hypertonic saline compared with samples induced by normal saline¹⁷ (intra-class correlation coefficient for neutrophils 0.6).

In summary, we have shown that repeated sputum induction within a 24 hour period leads to a rise in differential neutrophil counts in normal subjects. We conclude that sputum induction may have limited utility in serial assessment of airway inflammation within a 24 hour period.

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